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Title of research project:

Hypokinesia and circulation in the rat.

Hypothesis and objectives. Gravity has a profound effect on mammalian organisms. The effect of gravity on the cardiovascular system is especially great. It has been reported that during exposure of men to weightlessness certain circulatory adaptations occur. After return to earth new circulatory readaptations have been observed. The changes induced by weightlessness include shifts in blood distribution, overloading of atria, neurohumoral stimulation leading to excessive water loss and a profound blood volume decrease concomitant with other circulatory changes. These circulatory changes lead to orthostatic intolerance and a decreased work ability after return to earth.

Hypokinesia, especially antiorthostatic hypokinesia, mimics the effects of weightlessness. This has been shown in animal experiments for bone turnover (e. i. tibial bone formation rate, Morey, 1978 and 1979), nitrogen (Musacchia and Deavers, 1978 - 1980) energy (Jordan et al, 1979) and cation metabolism (Musacchia and Deavers, 1978) and in human experiments. Hypokinesia mimics in a high degree the effects of Og conditions on the cardiovascular system. Using an animal model that was developed in our laboratory and extensively studied and characterized as far as circulation is concerned both in our laboratory and in other laboratories in this country and abroad, we are presently studying circulatory mechanisms that occur during exposure to hypokinesia as well as during readaptation to control conditions. The cardiovascular measurements are performed in control experiments on unrestrained, unanesthetized rats and in the same animals in hypokinetic conditions or during readaptation to free activity. This part of our investigation serves to develop an adequate ground based model system as well as to identify possible circulatory mechanisms that evolved in mammals during many millennia of gravity exposure. It is hoped that the study will ultimately serve to predict and to identify possible circulatory mechanisms in animals exposed to conditions of Space Shuttle/Space Lab conditions.

Animals and techniques. Unanesthetized, unrestrained, resting Sprague-Dawley rats (200±10g) are used in all experiments. This is done because surgery and anesthesia drastically decrease cardiac output and other circulatory parameters in rats (Popovic, V. P. and Kent, K. M. Am. J. Physiol. 207, 767-770, 1964; Popovic, Pava, Popovic, V. P., Schaffer, R. and McKinney, A. S. Proc. Soc. Exp. Biol. 154, 391-396, 1977; Popovic, Pava, Schaffer, R. and Popovic, V. J. Neurosurgery 48, 962-969, 1978). Aorta and right ventricle of the animals are permanently cannulated fifteen to twenty-one days before the experiment. Arterial and central venous blood pressure (right atrium cannulated), intraventricular ECG, cardiac output, cerebral blood flow, and other cardiovascular parameters are measured with adequate techniques already developed and routinely used in our laboratory.

Hypokinesia. Holton's system for inducement of hypokinesia was initially used.

The system includes 1) the ability for the animal to exercise using only his front limbs, 2) a fluid shift, 3) total unloading of the rear limbs, 4) the ability to eat, drink, and groom, 5) a less stressful system thatn those presently existing, and 6) animals move about a 360° arc at will. The Musacchia modification (denim "astrosuit" instead of plastic harness) of antiorthostatic hypokinesia was used in all recent experiments. The head of the animals is tilted down in all experiments (20° - 30°).

Results. Most of our experimental work was in studying circulatory characteristics of rats during a 7 day long exposure to antiorthostatic hypokinesia and recovery to free activity (up to 30 days). Each experimental group had 8 animals of the same age, body weight, etc.

Cardiovascular studies. The resting mean arterial blood pressure was decreased during initial 48 hours of exposure to hypokinesia, while the heart rate of the rats was slightly increased. From the third day on the arterial blood pressure and the heart rate returned to the normal values. The right atrial pressure rose the first day of hypokinesia from 0 to +5 mm Hg. The second day the right atrial pressure declined and from the third day of hypokinesia on was at the same level as in control prehypokinetic conditions. The cardiac output of hypokinetic rats was increased the first day of exposure. The cardiac output decreased during the next three to four days until it was lower than in control animals. Stroke volume was increased during the early stages of exposure to antiorthostatic hypokinesia. After seven days of hypokinesia, cardiac output of rats was decreased during exercise on a treadmill (10m/min). Lactic and pyruvic acid accumulation was greater than in prehypokinetic conditions. The posthypokinetic animals were not able to maintain higher levels of exercise (20m/min). Cardiac output was lower after antiorthostatic hypokinesia in animals exposed to cold (0°C or -10°C external temperature) than in control animals. Of possible special interest were circulatory changes observed after administration of catecholamines. The antiorthostatic hypokinesia brought forth a profound decrease in responsiveness of peripheral vasculature. Dopamine effects were almost completely abolished while norepinephrine effects were much decreased. It seems that hypokinesia without head-down tilt does not lead to induced hypotensive responses.

Temperature regulation studies. The heat producing mechanism in animals after antiorthostatic hypokinesia are decreased. The heat dissipating processes are increased during hypokinesia but that might be the consequence of changes in posture and behavior, something Claude Bernard was aware already 120 years ago. The only significant difference between the Holton system and the Musacchia system was in temperature regulative abilities of the animals, especially dealing with the level of the temperature neutral (BMR) zone.

Conclusions. Our results indicate that antiorthostatic hypokinesia induces similar circulatory changes as seen in astronauts and cosmonauts after several days of exposure to 0g forces.

AIBS/NASA SPACE BIOLOGY SYMPOSIUM

2-3 October 1980

C O N T E N T S

Program

Abstracts (In numerical order of presentation as listed in the Program)

Attendees

PROGRAM

AIBS/NASA SPACE BIOLOGY SYMPOSIUM

Room B-239, Building 239
Ames Research Center, California

General Chairman

ROBERT W. KRAUSS
F.A.S.E.B.

P R O G R A M

	Thursday, 2 October 1980	*Abstract #*
8:30 a.m.	OPENING COMMENTS Thora Halstead, NASA Headquarters Harold P. Klein/Joe Sharp, Ames Research Center	
8:45 a.m.	INFLIGHT CONTROL AND RESEARCH CENTRIFUGE John W. Tremor, Ames Research Center	(1)
9:00 a.m.	RESEARCH ASSOCIATES TRAINING PROGRAM IN SPACE BIOLOGY X. J. Musacchia, University of Louisville	(2)

PLANT SYMPOSIUM

Session Chairman

ROBERT E. CLELAND
University of Washington

9:15 a.m.	AN EXAMINATION OF THE CLINOSTAT PROBLEM USING LEAFY PLANTS Frank B. Salisbury, Utah State University	(3)
9:45 a.m.	COFFEE	
10:00 a.m.	PHYSIOLOGICAL MODE OF ACTION IN MECHANICAL STRESS REGULATION OF PLANT GROWTH Cary A. Mitchell, Purdue University	(4)
10:30 a.m.	IDENTIFICATION OF A MISSING LINK IN THE TIME COURSE OF THIGMOMORPHOGENESIS IN BEANS Mark J. Jaffe, Wake Forest University	(5)
11:00 a.m.	INHIBITION OF GEOTROPISM AND ASSOCIATED CALCIUM REDISTRIBUTIONS BY A CALMODULIN-BINDING AGENT Stanley J. Roux, University of Texas, Austin	(6)
11:30 a.m.	MECHANISMS OF GRAVIPERCEPTION AND RESPONSE IN ETIOLATED PEA SEEDLINGS Arthur W. Galston, Yale University	(7)

12:00 Noon	LUNCH	
1:00 p.m.	PLANT CELLS, EMBRYOGENESIS AND MORPHOGENESIS IN SPACE Abraham D. Krikorian, State University of New York, Stony Brook	(8)
1:30 p.m.	EFFECTS OF GRAVITY AND WEIGHTLESSNESS ON THE DEVELOPMENT AND REGENERATION OF THE GIANT COENOCYTE CAULEPRA William P. Jacobs, Princeton University	(9)
2:00 p.m.	THE AMYLOPLAST AS A GRAVITY SENSING DEVICE IN PLANTS A. Carl Leopold, Boyce Thompson Institute	(10)
2:30 p.m.	THE GRASS PULVINUS-A SPECIALIZED GRAVITROPIC ORGAN Peter B. Kaufman and P. Dayanandan, University of Michigan	(11)
	GRAVITY RESPONSE IN THE GRASS PULVINUS IS CELLULAR ASYMMETRY THE ANSWER TO PERCEPTION? P. Dayanandan and Peter B. Kaufman, University of Michigan	(12)
3:15 p.m.	LOCALIZING AND IDENTIFYING GRAVITY SENSING MECHANISMS OF PLANTS Robert S. Bandurski, Michigan State University	(13)
3:45 p.m.	AROMATIC BIOSYNTHESIS IN HIGHER PLANT TISSUES Joe R. Cowles and H. W. Scheld, University of Houston	(14)
4:15 p.m.	PLANT MORPHOGENESIS AND PHYSIOLOGICAL BEHAVIOR IN RELATION TO GRAVITY Allan H. Brown, University of Pennsylvania	(15)
4:45 p.m.	AIBS SPACE BIOLOGY ADVISORY PANEL - EXECUTIVE SESSION	

Friday, 3 October 1980

ANIMAL SYMPOSIUM

Session Chairman

WILLIAM F. GANONG
University of California, San Francisco

8:00 a.m.	MAMMALIAN GRAVITY RECEPTORS: STRUCTURE AND METABOLISM Muriel D. Ross, University of Michigan	(16)
8:30 a.m.	DEVELOPMENT AND FUNCTIONAL DIFFERENTIATION OF VESTIBULAR SYSTEM IN NORMAL, ROTATED AND CENTRIFUGED RATS AND MICE J. Richard Keefe, Case Western Reserve University	(17)
9:00 a.m.	AVIAN EMBRYONIC DEVELOPMENT IN HYPERDYNAMIC ENVIRONMENTS U. K. Abbott and A. H. Smith, University of California, Davis	(18)

9:30 a.m.	ESTABLISHMENT OF THE DORSAL/VENTRAL POLARITY OF THE AMPHIBIAN EGG--USE OF EGG ROTATION RE. GRAVITY AND ULTRAVIOLET IRRADIATION AS PROBES George M. Malacinski, Indiana University	(19)
10:15 a.m.	COFFEE	
10:30 a.m.	A GROUND-BASED COMPARATIVE STUDY OF THE EFFECT OF WEIGHTLESSNESS ON THE DEVELOPMENT OF AMPHIBIAN EGGS George W. Nace, University of Michigan	(20)
11:00 a.m.	DROSOPHILA BIOLOGY IN SPACE Jaime Miquel, Ames Research Center	(21)
11:30 a.m.	THE VISUALIZING OF EQUILIBRIUM RECEPTOR AFFERENTS AND INTERNEURONS BY HRP AND COBALT CHLORIDE HISTOCHEMISTRY Becky J. Anderson and H. Bernard Hartman, Texas Tech University	(22)
12:00 Noon	LUNCH	
1:00 p.m.	THERMOREGULATION IN RATS: EFFECTS OF VARYING DURATION OF HYPERGRAVIC FIELDS J. M. Horowitz and B. A. Horwitz, University of California, Davis	(23)
1:30 p.m.	HYPER-GRAVITATIONAL EFFECTS ON METABOLISM AND THERMOREGULATION Jiro Oyama, Ames Research Center	(24)
2:00 p.m.	GRAVITY AND BODY COMPOSITION IN THE RAT Grover C. Pitts, University of Virginia School of Medicine	(25)
2:30 p.m.	COFFEE	
2:45 p.m.	GRAVITATIONAL SCALE EFFECTS Nello Pace, Donald F. Rahlmann and Arthur H. Smith, University of California, Berkeley	(26)
3:15 p.m.	STRUCTURAL DEVELOPMENT AND GRAVITY Emily R. Morey-Holton and Thomas J. Wronski, Ames Research Center	(27)
3:45 p.m.	HYPOKINESIS AND ANTIORTHOSTASIS; AN ANIMAL MODEL FOR STUDIES OF PROBLEMS INVOLVED IN WEIGHTLESSNESS X. J. Musacchia, Dan Deavers and Joseph Steffen, University of Louisville	(28)
4:15 p.m.	HYPOKINESIA AND CIRCULATION IN THE RAT Vojin P. Popovic, Emory University Medical School	(29)
4:45 p.m.	ADJOURN	

ABSTRACTS

D1

Variable Gravity Research Facility (VGRF)

John W. Tremor
Robert W. Mah

Ames Research Center

The Agency has for some time been aware of the desirability of providing an on-board centrifuge for Spacelab Life Sciences experiments. The requirement for both a 1 g control and a hypogravity (0-1 g) capability is expressed in the NRC Space Science Board's reports of 1974 (Scientific Uses of the Space Shuttle) and 1979 (Life Beyond the Earth's Environment). Also, the Life Sciences Advisory Committee of the NASA Advisory Council in its 1978 report on Future Direction for the Life Sciences in NASA, pointed out that the dearth of proper controls has been a significant cause for the controversial results of previous space biology experimentation. The lack of a 1 g on-board control is strongly implicated in this criticism. In response to this generally felt need, NASA has conducted a study of a general purpose Spacelab Bioresearch Centrifuge and, more recently, examined requirements for expanding the usefulness of the Vestibular Research Facility (see figure). These studies, although conducted without the direct participation of the scientific community, anticipated the probability that many questions will remain unanswered by flight experiments that are relatively uncontrolled for the g variable.

We are now supporting a number of prospective investigators who have a clear and firm requirement for an inflight 1 g control, to the point in some cases of calling for an experiment unique centrifuge if nothing else is available. Too, like requirements exist for another set of investigators, those working in the field of Gravitational Biology, a discipline long supported by NASA. These experimenters, currently active in describing the dynamics of biological response to hypergravity, are vitally interested in fields of g between 1 and 0 to determine continuous or threshold effects.

A Science Advisory Group of investigators has been formed, along with Headquarters and Ames Research Center staff, to define the various parameters influencing centrifuge design, e.g., species and numbers to be accommodated, rate of centrifuge onset and stopping, tolerance of centrifuge stoppage, length of arm and coriolis sensitivity, constraints of life support systems, value and number of hypogravic levels, g gradients tolerated, permissible vibration levels, etc. An SR&T task has been initiated to implement the requirements forthcoming from workshops and advisors. This effort will specifically involve a modification of the present basic design of the VRF centrifuge. A prototype facility will be fabricated and tested and lead ultimately to flight hardware.

D2

Research Associates Training Program

in Space Biology

X. J. Musacchia

University of Louisville, Louisville, Kentucky

Objectives of the Program

The objective of this program is to involve prospective young scientists in ongoing programs in NASA Space Biology projects. The Space Biology Program, currently undergoing an orientation of development focused on the newly evolving Shuttle program, presents the biological community with one of the most dynamic and opportune programs in the biological sciences. Heretofore, there has been little opportunity to conduct research in gravitational biology (i.e. weightlessness).

The young scientists selected for this program would come from newly trained doctoral degree recipients and would be in a position to provide full-time effort in established research programs which are oriented in space biology objectives. They will participate in the research programs projected for the immediate future. During the next decade the Shuttle program will begin its emergence as the main vehicle for outer space research and it is critical that a cadre of emerging researchers be involved in the initial stages of research developments. It is a premise of this proposal that their involvement is most efficiently developed within ongoing research programs oriented in Space Biology.

Research Possibilities

This program stresses the research opportunities which provide individuals an experience which can enhance their immediate professional objectives. They would be selected for ability and desire to work side by side with scientists who have demonstrated their laboratory research commitments to NASA's Space Biology Program, i.e. laboratories that are currently supported by NASA through the Space Biology Research Program.

Space Biology Interactions

A portion of the program would be designed to encourage these individuals working in university NASA sponsored research projects to visit, from time to time, NASA research centers in order to participate in on-going programs. In addition, there are plans to conduct an annual meeting in order to bring these research colleagues together for workshops and symposia. Featured participants of such programs would be personnel selected from NASA research centers and university centers with space biology related projects. The overall effort would be to create a current and viable exchange of ideas.

Selection/Project Review Committee

Investigators will be selected on a competitive basis from applications which will include a description of a research protocol, the qualifications of the candidate and the statement of his or her proposed aspirations. Selections will be made by an extramural Project Review Committee. Periods of support would range for not less than six months and not more than 12 months and with an option for renewal. The renewals would be reviewed by the Project Review Committee and selection and approval will rest on the research productivity and the research potential demonstrated during the tenure of the support.

The Project Review Committee will meet twice a year to examine the applications and to make the recommendations for awards. Selections and notifications will be made two times annually.

Current Status

The program was announced in several journals during the summer, 1980, i.e. The Physiologist, BioScience, Federation Proceedings and Science magazines. Applications have been received and processed.

Thirty NASA funded Space Biology laboratories were asked if they would want to participate in the program. Of these, eighteen have submitted descriptions of their research and agreed to host an Award candidate.

For the current year of funding, twenty-four people have requested application forms. If they apply for an Award, they must submit a research proposal and obtain an agreement from the laboratory of their choice. These are due October 25. From these seven or eight people will be given Research Associate Awards. The awards will be initiated early in November, 1980.

AN EXAMINATION OF THE CLINOSTAT PROBLEM USING LEAFY PLANTS

NASA Project NSG-7667

It has been suggested that clinostat epinasty (downward curvature of leaves) is a response to the bending and flopping of leaves rather than to gravity compensation. The epinasty is most likely caused by ethylene, as we confirm by delaying its development with an inhibitor of ethylene synthesis, aminoethoxyvinylglycine (AVG), and with two inhibitors of ethylene action (Ag^+ and CO_2). During the past year we have developed equipment to measure small amounts of ethylene from intact plants; we hope to relate ethylene production to development of epinasty on the clinostat.

As reported last year, our attempts to induce epinasty in vertical plants by gentle mechanical stresses (horizontal or vertical shaking, twisting, rubbing, bending, etc.) have failed to cause epinasty. In a further test, plants were placed upright on the turntables of our clinostat. Once every four minutes (normal clinostat rotation time), we tipped the clinostat so stems were horizontal and rotated plants once in 10 s, after which plants were returned to the vertical, the entire operation requiring about 20 s. Thus, plants were subjected to leaf flopping closely similar to that occurring on clinostated plants but for such a brief interval that gravity perception was probably not upset. No epinasty developed. On the other hand, we can induce epinasty with a minimum of leaf flopping by inverting plants at 10-to 30-min intervals, leaving them upside-down half the time (controls inverted and immediately returned, as reported last year).

We conclude that clinostat epinasty is probably a response to gravity compensation (i.e., upsetting of gravity perception), rather than the mechanical stresses of leaf flopping. Thus, it is important to understand the nature of gravity perception in leaf blades and petioles as well as in stems. Although our search is only beginning, we so far fail to see obvious statoliths in the cells of our light-grown leafy stems. We do measure up to 18% starch in both leaves and stems, but the measurements need to be repeated. We also need to see if some clinostat symptoms are caused by mechanical stresses. Stem growth is strongly inhibited on the clinostat and could be such a symptom.

In some ways it is simpler to study bending of stems laid horizontally than reorientation of leaves, although both are examples of gravitropism. (Leaf position is also influenced by daily sleep movements.) Raymond Wheeler has somewhat extended results reported last year, indicating that ethylene plays a primary role in the gravitropic bending of stems. Bending rates are delayed by the ethylene inhibitors mentioned above, plus Co^{2+}, which inhibits ethylene synthesis.

Much of our work during the past year has investigated the restricted gravitropic response, in which plants are restricted to the horizontal position for several hours and then released. Bending occurs within 1 to 10 s after release, suggesting that bending energy is stored in restricted stems. Bending will continue for another 2 h or so after plants have been returned to the vertical, suggesting that bending stimulus is also stored. Ethylene inhibitors reduce

storage of bending energy but do not influence storage of stimulus.

We have taken two approaches to an understanding of the restricted gravitropic phenomenon. Wesley Mueller has measured the changes in surface dimensions of stems, using a refined technique of stereo photography that allows a "freezing" of stem dimensions in time. He finds that bending plants grow about the same on the lower side of the stem as do comparable nodes on vertical plants, but growth of the upper side of bending plants is greatly reduced. When plants are restricted and then released, the upper side shrinks as much as 40%, while the bottom expands to about 110% of restricted length.

Julianne Sliwinski has measured changes in cellular dimensions. Her preliminary results show that cells on the bottom of restricted stems are thicker, and those on the top are thinner, than cells of vertical controls. After release of the stem from restriction, cells on top shorten and thicken, while those on the bottom lengthen and become thinner. Thus, bending occurs as cells on both top and bottom conserve their volumes but change their relative lengths and diameters.

The time-honored Cholodny-Went theory suggests that gravitropism occurs as auxin moves to the bottom of stems placed horizontally, causing increased growth there. Our results show no increase in growth rate for bottom cells compared to vertical controls but show a clear inhibition of growth in top cells. Indeed, in restricted plants, growth of top cells stops almost completely so the increased length during restriction is caused by an elastic stretching of the cells. It seems possible that inhibition occurs on top in response to ethylene. We need to examine relative ethylene production from the top and the bottom of stems placed horizontally.

The fact that cells on the top shrink while those on the bottom elongate after release from restriction suggests that cell-wall microfibrils have been laid down during the restriction period in a manner that will control these subsequent dimensional changes. This must be true for the bottom cells, at least, although it is conceivable that top cells stop all microfibril deposition shortly after being placed horizontally. Thus we need to examine microfibril orientation. Michael Walsh, a plant anatomist on our campus, will cooperate with us in this effort during the coming months.

The current theory of cell growth suggests that plant cells take up water osmotically as the cell wall "loosens" (becomes more easily stretched plastically), lowering the internal pressure and thus the internal water potential. But in restricted stems, pressure builds on the bottom of the stem as tension (stretching) builds on top. How can we account for cell growth and potential for growth under conditions of increasing cell pressure? It is important that we examine the actual magnitudes involved. Thomas Blotter in the Department of Mechanical Engineering is cooperating with us to measure actual forces exerted by the bending stems and thus the internal compression.

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Physiological Mode of Action in Mechanical Stress Regulation of Plant Growth

Principal Investigator: Cary A. Mitchell

Plant growth responses to periodic gyratory shaking generally include reductions in internode elongation, dry weight gain, leaf area, and node and internode diameter in a number of herbaceous species. Shaking increases succulence and density of leaves in tomato. Greenhouse-grown plants lose much of their sensitivity to mechanical stimuli during summer months, and regain it during winter months. Outdoor-grown plants lose virtually all sensitivity (in terms of growth reduction) to moderate shaking treatments. Because of the seasonal differences in mechanical sensitivity, presumably due to uncontrolled environmental fluctuations, we have developed six small controlled-environment plant growth units, several of which have been fitted with vibrators. Plants can be grown and mechanically-treated in these units without disturbing the plant growth environment. Under conditions of constant CO_2 level, high humidity, and moderate light and temperature levels, tomato seedlings grew rapidly, but stem elongation, density, and dry weight gain were enhanced by 30 minutes per hour of low amplitude (< 0.1 mm) vibration of about 3000 cpm. Vibrated stems were less succulent than those of controls. Vibrations also enhanced area and succulence of leaves, but reduced leaf density. Low humidity (< 50% RH) in the flowing atmosphere greatly reduced plant growth relative to that at high humidity (≥ 90% RH). Vibration at low RH stimulated leaf H_2O content and stem fresh and dry weights. Difficulties were encountered in finding a reliable root support medium for soilless culture within the controlled-environment units. A number of media were surveyed and all were found to have particular problems. Therefore, a recirculating solution culture system is being developed for incorporation into the plant growth-vibration units.

Shaking stresses also reduce net photosynthetic productivity in several herbaceous species. Growth analyses with soybean suggest that photosynthetic efficiency as well as photosynthetic surface area were affected by mechanical treatment. However, distribution of dry weight was not altered by shaking. Both the growth as well as the mechanical sensitivity of soybean plants was reduced when grown outdoors. Although outdoor-grown plants were very stunted relative to indoor controls, they accumulated more dry weight than did the unhardened indoor plants, largely due to more extensive root growth. Greenhouse plants shaken continuously during development were not shorter than undisturbed controls, but did accumulate much less dry weight in both roots and shoots. Soybean plants were grown and shaken at 5 different light levels in a greenhouse in the summer in an effort to mimic winter lighting conditions and to explain seasonal stress sensitivities. Stem length was most sensitive to mechanical stress at 24% of full light, and total dry weight was most sensitive at 31%. At 17% full light, stems of undisturbed plants were too weak to support shoot weight, but shaking treatments strengthened stems enough to allow them to remain erect.

Shoot strengthening aspects of mechanical stress are being examined with young, greenhouse-grown tomato plants subjected to a minimal number of shaking treatments (10 days) in which only a few growth parameters have yet responded in the winter and none in the summer. Longitudinal modulus of elasticity was greatly increased in hypocotyls in the winter as a result of shaking, but only slightly in the summer. Stress mildly strengthened first internodes in the winter, but not in the summer. Fiber analysis of stem and petiole tissues of stressed and unstressed plants grown at different seasons of the year is in progress.

D5

M. J. Jaffe, Biology Department, Wake Forest University, Winston-Salem, North Carolina.

A. IDENTIFICATION OF A MISSING LINK IN THE TIME COURSE OF THIGMOMORPHOGENESIS IN BEANS. Both mechanical perturbation, in the form of rubbing, and wounding cause ethylene evolution from bean internodes. This evolution begins 45 to 60 minutes after perturbation or wounding and peaks about 2 hours later. Maximal thigmomorphogenesis occurs when the internodes are perturbed when they are 10 mm or less in length. Maximal ethylene evolution, however, occurs in slightly longer internodes. When one internode is perturbed, ethylene evolution was not observed in other internodes even though they respond thigmomorphogenetically. Ethylene evolution is apparently a result of the increased production of cyclopropane-1-carboxylic acid (ACC) since this occurs during the lag period preceeding ethylene evolution. Inhibitors of ACC and ethylene synthesis block increased radial growth but not reduced elongation. Ethylene may therefore be only one of several factors causing thigmomorphogenesis. Future work will attempt, on the one hand, to see how membrane changes induced by mechanical perturbation cause ACC production, and on the other hand, how the ethylene trigger induces cell division in the secondary xylem and pith.

B. THE USE OF A PEA MUTANT AS A PROBE TO STUDY GRAVITROPISM. The pea mutant "ageotropum" is being used to study gravitropism. The <u>ageotropum</u> stem is not gravitropic in the etiolated state, but begins to grow upward when it is illuminated. Although phototropism may also be involved, the evidence thus far suggests that the stem becomes sensitive to gravity when the

photosynthetic apparatus begins to develop. Thus, it is possible that the part of the gravitropic mechanism that is deleted by the mutation can be restored by photosynthesis.

Inhibition of Geotropism and Associated Calcium Redistributions by a Calmodulin-Binding Agent

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Department of Botany
The University of Texas at Austin

Introduction and objectives: When eucaryotic cells translate external stimuli into physiological processes, they often do so by cellular mechanisms that include as an early step transient changes in the cytoplasmic concentration of calcium. Such changes alter the activity of the calcium-binding protein, calmodulin, a key regulator protein that has been shown to control the function of one or more critical enzymes in all eucaryotic cells tested to date. Recent papers have shown that calcium is redistributed asymmetrically between the concave and convex sides of geotropically stimulated hypocotyls and coleoptiles. This redistribution seems to precede the development of curvature, and agents that block geotropism also block the calcium redistribution. We recently found that an oat plasma membrane Ca-ATPase, which hypothetically could play a role in the active redistribution of calcium across geotropically stimulated tissue, is activated by calmodulin from oats. Given this background information, we decided to test whether we could: (1) confirm and extend the observations of others on the redistribution of calcium across geotropically stimulated tissue; and (2) inhibit this calcium redistribution, and concomitantly geotropism, with the drug chlorpromazine, an agent known to bind to calcium-activated calmodulin and block its interaction with target enzymes, such as the plasma-membrane CaATPase from oats.

Methodology: The distribution of ^{45}Ca between the concave and convex sides of two-cm-long coleoptile segments taken from the curved region of geotropically-stimulated corn seedlings was measured three hours after the plants had been placed in a horizontal position. The segments were weighed, dried, solubilized in Brays' solution, then measured for beta radioactivity. The cross-sectional distribution of ^{45}Ca in one-mm-thick sections of geotropically curved corn coleoptiles was measured by autoradiography. These sections were taken, three hours after the geotropic stimulus was given, above, through, and below the curved region of the coleoptile. The radial and longitudinal distribution of calcium in the coleoptiles and primary leaves of three-day-old oat seedlings was examined by light and electron microscopy after the samples were treated with potassium pyroantimonate to precipitate calcium *in situ* as large, electron-dense aggregates. Using this method, we examined both chlorpromazine-treated and untreated samples.

Results to date: Intact seedlings treated with 10^{-5} to 10^{-6}M chlorpromazine for six hours show essentially no geotropic curvature three hours after being placed in a horizontal position, whereas untreated controls show significant curvature by this time. The overall elongation growth of treated and untreated samples is about equal. Twelve hours after geotropic stimulation, treated seedlings show a slight upward curvature broadly distributed along the length of the plant; but untreated seedlings show a sharp upward curvature localized in a relatively narrow region 2-3 cm below the tip. We found, in agreement with earlier studies, over 4X more ^{45}Ca per unit dry weight or fresh weight on the concave (slower-growing) side of curved corn coleoptile segments than on the convex side. Autoradiograms of one-mm-thick cross sections showed that this asymmetry was restricted to the curved region only and was not detectible in the straight regions above and below the curve of the coleoptile.

The autoradiographic results were confirmed and extended when calcium was localized by the pyroantimonate method. Using this technique, we found that chlorpromazine-treated seedlings did not show either an asymmetric distribution across the coleoptile nor any curvature

2.5 hours after being placed in a horizontal position. We also found that the build-up of calcium on the perspective concave side of untreated seedlings began before there was any clear evidence of curvature and that this build-up was concentrated preferentially in the epidermal cells of the coleoptile. No asymmetric calcium distribution was detected in cross sections taken from curved (or straight) regions of the primary leaf of seedlings that had undergone 2.5 hours of geotropic growth.

Conclusions: It seems likely that the preferential concentration of calcium in the epidermal cells on the concave side of coleoptiles is a necessary step in the transduction of the gravity stimulus into geotropic growth. The data do not support the idea that the calcium redistribution is a sufficient event to promote geotropic growth; and, in fact, others have shown that this is probably not so. The chlorpromazine data are consistent with the hypothesis that calmodulin may play a role in mediating geotropism, probably through its influence on plasma-membrane CaATPases. The observation that calcium is concentrated in the epidermal cells on the concave side of geotropically curved regions of coleoptiles is consistent with current models that ascribe a special role to outer cell layers for mediating geotropic growth.

Research in Progress: Our current experiments include:

a) Completion of our electron microscopic examination of calcium redistribution in geotropically stimulated oat seedlings.

b) An examination of the influence of chlorpromazine and other phenothiazines on the ability of intact cells to pump calcium out across the plasma membrane.

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Mechanisms of graviperception and response in etiolated pea seedlings

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Introduction

Last year, we presented three kinds of evidence against a compulsory linkage between circumnutation and gravitropism. (1) Circumnutation in etiolated pea seedlings describes an elliptical path, with the long axis in the plane bisecting the apical bud, and very restricted movement at right angles of this plane, yet gravitropic experiments in the two different planes showed identical presentation times and kinetics of response. (2) Totally etiolated pea seedlings do not circumnutate at all, yet they respond well in gravitropism. Red light, which increases the frequency and amplitude of circumnutational oscillation, has an effect on gravitropic presentation time, not on kinetics of early response in intact seedlings. Previous reports of red light potentiation of gravitropism were shown to be erroneous, in that previous workers had used cut sections for their experiments. We showed that cutting inhibits gravitropism, probably via wound generated ethylene, and that red light absorbed by phytochrome annuls the effect of injury by inhibition of such ethylene release. (3) Decapitated seedlings supplied with auxin do not circumnutate, yet they react well gravitropically. (4) This year, we have obtained additional evidence separating circumnutation and gravitropism through the use of naphthylphthalamic acid (NPA), a known inhibitor of polar auxin transport. This substance inhibits circumnutation at concentrations (ca 5 μM) that have no effect on growth or gravitropic response.

Bundle sheath amyloplasts as statoliths

1. Displacement of bundle sheath amyloplasts by gravity.

Our experiments this year have concentrated on amyloplasts, the presumed receptors of the gravitropic stimulus. Using both light and electron microscopy we have examined various zones of six-day old etiolated Alaska pea seedlings. Using thick hand-cut sections from fresh material and I_2-KI staining, we were able to observe many small starch grains, mainly in the cortical cells, and larger densely staining starch grains in the bundle sheath. The larger starch grains in the bundle sheath cells sedimented within five to ten minutes after gravitropic presentation, while the smaller starch grains in the cortex did not. Thus, the multigranular amyloplasts of bundle sheath cells are presumed gravitropic receptors, as suggested by others.

2. Correlation between starch content and gravitropic behavior.

Previous investigators had attempted to deplete gravitropic organs of their starch by hormonal treatments, mainly involving cytokinins and gibberellins. We found such treatments completely ineffective in peas. However, we were able to achieve significant alteration of starch content and of gravitropic sensitivity by techniques involving cold storage and cotylectomy. It is well known that in the potato tuber, starch is massively hydrolyzed to sugar when the organs are stored between 2° and 5° C. Accordingly, we transferred etiolated pea seedlings to a cold room maintained at 3-4° C for varying periods.

The seedlings were then removed, an aliquot removed for histological examination for starch, and the others subjected to gravitropic stimulation. Within three days of cold storage, both starch content and gravitropic response manifested at higher temperatures was significantly inhibited; after one week, response was approximately 50% of the controls. Cotylectomy prior to placement in the cold room significantly increased the rate at which both starch and gravitropic response disappeared, and the two parameters were roughly parallel. Cotylectomy also significantly reduced (by about 65%) straight growth manifested after transfer to higher temperatures.

Plants stored in the cold showed no irreversible damage, either to starch synthesis or tropistic sensitivity by virtue of one week of cold storage. Thus, plants stored for one week in the cold with or without cotyledons regain virtually all of their gravitropic sensitivity and starch after 4 hrs at the higher temperatures of the growth room.

Fine structure of statolithic amyloplasts and surrounding regions of the cell.

We studied the fine structure of multigranular amyloplasts in a Phillips 300 electron microscope after glutaraldehyde fixation, embedding in methacrylate or Spurr's, sectioning in an LKB ultramicrotome at 60-90 nm (light gold), and double staining with uranyl acetate and Reynolds' lead citrate. Sections from stems kept erect or inclined 90° to the vertical showed close approximation of the multigranular amyloplast with the plasma membrane and usually with the endoplasmic reticulum near the bottom of the cell. In most instances it appears that the amyloplast need not be in contact with the plasma membrane, but rather with ER and other elements of the cytoskeleton close to the physiological bottom of the cell.

Phytochrome and starch metabolism.

Red light perceived by phytochrome results in a rapid and massive breakdown of starch, but not in the multigranular amyloplasts. This is shown both microscopically and by chemical determination of residual starch and reducing sugars. Thus any effects of red light on tropistic behavior are not correlated with changes in the statoliths.

Conclusions

The multigranular amyloplasts of the bundle sheath correlate well with the presumed statolithic gravitropic receptors in that: (1) They sediment during appropriate gravitational presentation times, (2) Their depletion in the cold and by cotylectomy correlates well with decreasing gravitropic sensitivity, (3) Their return after transfer of previously cold-depleted plants to warm rooms parallels the return of gravitropic sensitivity, and (4) Their close contact with the plasma membrane, ER and other cytoskeletal components at the bottom of the cell provides some indication of systems with which they could interact.

Plant Cells, Embryogenesis and Morphogenesis in Space,

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The basic principles and procedures of our investigations have been summarized a number of times and will only be briefly alluded to here. Higher plants provide materials with which to ascertain a) the stimuli that release the otherwise suppressed totipotency of mature quiescent cells as they exist in the plant body; and b) the factors that control the direction and pace of their subsequent development. Because of the known responsiveness of plant growth and development to a variety of external factors, the space environment introduces into these considerations, an entirely new set of conditions, the consequences of which are, as yet, largely unexplored. The general aim of our research is to design and perfect test systems using green plants at different levels of initial organization which, because they are capable of further growth and development, may be exposed to the effects of the near-zero gravity environment. In this way their behavior and growth and development in space may be compared and contrasted with their performance in 1 g controls. Working with plants at the level of free cultured cells, free protoplasts, derived embryos and plantlets, we anticipate disclosing the degree of organization at which salient problems of the space environment may arise. Questions to be asked are, whether plant development can proceed normally in the weightless or near-weightless state--especially whether isolated cells can give rise to embryos and plantlets; can the processes of nutrition and metabolism occur normally in plant cells and organs that are not actually subjected to the gravitational stimulus and, are plant physiological functions unaffected in cells and organs that have not only developed in weightlessness but may be regarded as having no long-term "memory" of actual contact with gravity? In addition to its fundamental importance for space biology, the work helps us to achieve a new and better understanding of the controls of plant development.

In the past year, we have spent a significant portion of our time developing a model monocotyledonous system based on daylily (Hemerocallis). One special feature of this system is that like the carrot we can work with totipotent free cells, but unlike carrot, it is possible to carry out cytological analysis of the chromosomes. Another highly desirable feature is that the daylily is a perennial plant and continuously renews its capacity for vegetative growth by means of activity in basal meristems. Since we already have established that carrot is a useful test organism and admirably serves as a test or model dicotyledonous system, it has been satisfying that our selection of daylily as a "model monocot" is likewise proving to be amenable to space experimentation.

The major portion of the presentation will deal with establishing and defining the totipotency and the clonal nature sensu stricto, and hence the exceptional usefulness of daylily, but it will be shown that work on carrot cells and protoplasts also continues. The results of K302 carrot cell experiment of Cosmos 1129 are still being evaluated but the outcome of that experiment thus far will be summarized. Similarly, our work on Arabidopsis is still in progress, breeding of a dwarf line is in progress and our studies are permitting us to controllably use it.

Our work with daylily protoplasts thus far suggests that it will be of special interest to establish whether protoplasts from select diploid cell lines can be fused in a controlled way with other diploid protoplasts. The events associated with the production and growth of absolutely isolated single protoplasts

and their significance for experimentation in plant biology in space are such that it could well serve as a model for simulated "fertilization" *in vitro*. Since we are concerned with ascertaining whether fertilization could occur in hypogravity conditions, there is every reason to believe that meaningful correlations could be made by studying the fusion process in a hypogravity environment.

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NASA Grant NSG-7280: "The effect of gravity and weightlessness on the development and regeneration of the giant coenocyte Caulerpa."

Caulerpa is a marine alga with unusual internal structure: although it has three different types of "organs" (leaves, rhizomes, and rhizoids), each with a different orientation to the environment, the organism is a giant, multinucleate, single cell. The coenocytic structure of this large cell offers unique opportunities for experimental work on the basis of gravity responses.

We have found that gravity alone can control the location of rhizoid-development near the tip of the elongating rhizome (Jacobs & Olson, 1980). Turning the plant upside down by rotating the rhizome 180° around its horizontal axis causes all subsequently developing rhizoids to develop on the "new-underside" of the rhizome and new leaves to develop on the "new-upperside". This fast switch under the influence of gravity in the location of organ differentiation occurs with no significant change in the rate at which these organs differentiate nor in the rate at which the organs elongate. Discovery of the internal factors controlling this fast, gravity-influenced change in rhizoid-differentiation is now our major interest.

We are following two main lines of investigation: 1) To identify gravity sensors inside the cell; 2) To establish how their effects are mediated. We are using light- and electron-microscopes to see what internal particles sediment quickly enough under the influence of gravity to be logical candidates for the roles of gravity sensors. Then we insert magnetic particles, as artificial gravity sensors, into the giant cell with the aim of testing the hypothesis that organelles that act as gravity sensors in plants act by exerting mechanical pressure on membranes.

There is much evidence from the multicellular vascular plants that hormones are involved in gravity responses. I have shown that indole-3-acetic acid, a well-known hormone of vascular plants, induces rhizoid formation in Bryopsis, a coenocytic alga related to Caulerpa but not so large or so differentiated. Bioassay activity like that of indole-acetic acid was shown in extracts of Bryopsis, and gibberellin-like activity has been demonstrated in Caulerpa extracts by Augier. We have found that adding gibberellic acid to Caulerpa produces a striking increase in elongation. During the past spring we have started experiments to identify with mass spectrometry the native gibberellin (or gibberellins) and other hormones of Caulerpa. (No one has as yet identified the chemical structure of any gibberellin from any alga, so such identification will be of special interest from the point of view of comparative biochemistry, as well as valuable for our Caulerpa research.)

Publications this year:

1) Jacobs and Olson. (1980). Developmental changes in the algal coenocyte Caulerpa prolifera (Siphonales) after inversion with respect to gravity. American Journal of Botany 67: 141-146.

2) Brennan and Jacobs. (1980). Polarity and the movement of [^{14}C]indol-3-ylacetic acid in the coenocyte, Caulerpa prolifera. Annals Botany 46: 129-131.

D10

THE AMYLOPLAST AS A GRAVITY SENSING DEVICE IN PLANTS

Abstract

NASA Grant NSG 7657

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Gravity sensing tissues of plants are almost invariably associated with starch-bearing plastids, the amyloplasts. A precise role of amyloplasts in gravity sensing is widely accepted, on the basis that their high density (1.5) makes them the most rapidly moving cell component upon gravity stimulation, and the observation that variations in size alter their gravity sensing in a manner predicted by Stokes Law (Hertel et al., 1969). While there is agreement that the amyloplast is the most usual gravity sensing component (Wilkins, 1977; Juniper, 1976), there is no understanding of how the movement of these organelles might bring about a physiological gradient across a cell or tissue. A major objective of this study has been to examine the amyloplast itself and to attempt to define means by which movement of the organelle could bring about a physiological gradient.

As a first step in this study, we have prepared intact amyloplasts from several geosensitive tissues. The extreme fragility of amyloplasts may well be a consequence of their enclosure of large, heavy starch grains in a fragile membrane envelope; but attempts to isolate them from aqueous preparations end up with a large proportion of broken amyloplasts. As an alternative we have utilized a non-aqueous method adapted from the work of Liu (1979). Coleoptiles or root tips of corn are frozen in Freon-12, lyophilized and ground in cold hexane. A fracture plane falls preferentially around the organelle and the fragments sieved (15 μm) and fractionated in hexane give a good yield of amyloplasts. Electron and light microscopy confirm the presence of intact amyloplasts. The extent of contamination of the preparation with other cell components was defined by marker enzymes (pyruvate kinase for cytosol and glutamate dehydrogenase for mitochondria).

A second approach to defining the nature of amyloplast action has involved measurement of the surface charge on the organelle. Intact amyloplasts are released from fresh tissue by chopping and sieving through a 10 μm sieve, and the organelles are electrophoresed using a microscopic electrocataphoresis cell. We have found that there is a negative surface charge on the amyloplast, and work in progress will characterize the zeta potential and hence permit the estimation of the nature and magnitude of the surface charge. In contrast to the negative charge on the amyloplast, the starch grains themselves have a distinct positive charge.

A third approach is to investigate the extent of microfilament (actin) networks in geosensitive cells on the possibility that the movement of the amyloplasts through the cytoplasm may serve to bring them in contact with

such an endoskeletal feature of the cytoplasm. Inconsistencies in the kinetics of geosensing and the current concept of statoliths resting on the plasmalemma of the cell (Johnsson and Pickard, 1979) might be resolved if reports of the presence of microfilaments in plant cells (Farere and Jackson, 1975) prove to be correct. We are attempting to observe such filaments in geosensitive tissues using a fluorescently labeled fungal toxin (phallicidin) which binds to microfilaments with high specificity (Barak et al., 1980).

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THE GRASS PULVINUS-A SPECIALIZED GRAVITROPIC ORGAN

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The grass pulvinus is an organ uniquely specialized for gravity response. We have studied the structure of the grass pulvinus in more than 25 species of grasses and the gravitropic response in some detail in oats. The gravity-sensing organ is a swollen portion of the leaf base, just above the node. In the panicoid grasses, such as corn and sorghum, another gravity-sensing region is located at the base of the internode. Both these pulvini are quite similar morphologically and both possess statoliths. The developmental history and the mature structure of the pulvinus point to its being a very sensitive gravity sensor to keep the plants erect. The pulvinus is unsilicified in comparison with the rest of the shoot system. Where some silicification does occur in the form of silica cells and trichomes, the extent of silicification is very poor. Lignification is limited only to few vascular elements in this region. The bundle cap, which is highly lignified in regions above and below the pulvinus, are unlignified and collenchymatous in the pulvinus. Statenchyma, with readily sedimentable starch statoliths, occur in the pulvini.

We have studied the gravitropic response of the pulvinus using excised segments. The responses have been characterised with respect to length and age of the segment, temperature and osmotic requirements, response to plant hormones and inhibitors, and species. Using an angular displacement transducer and a continuously recording of the growth response, we have also studied the growth kinetics.

The pulvinus seems to be a self-contained system. Deletion of the shoot on either side of the pulvinus does not interfere with the bending response. Excessive weight beyond the pulvinus depresses the degree of bending. The response of the pulvinus is age-dependent. Very young pulvini and older ones respond poorly. The older pulvini become less responsive, partly due to increasing lignification, and to a lesser extent, to increasing silicification. Starch statoliths sediment completely within 10 to 15 minutes of gravity stimulation. Statoliths are found in every pulvini examined in every plant, including a lazy mutant of corn. Among the grasses studied, Bromus inermis (brome grass) shows the poorest gravitropic response and Hordeum vulgare (barley) the best response.

On horizontal placement of a segment, a negative gravitropic response starts between 20 and 50 minutes, and growth continues for the next several days. During this period, an 180 degree reorientation of the segment initiates growth on the now lower surface. Such alternate orientations can make the pulvinus grow nearly four times the original length.

Growth is strictly by cell elongation. All of the cells in the pulvini elongate; but, the elongation of the collenchymatous cells is most unique. They stretch in specific regions only, leading to a pattern of alternately thick and thin regions, as seen in a polarizing microscope. Elongation of the pulvinus can also be brought about by the application of auxins. We have also found that gibberellic acid participates in the gravitropic response. Inhibitors of auxin transport, such as TIBA and CFM, interfere with the growth response of the pulvinus.

We have analysed the native IAA and GA contents of the oat pulvinus. When oats are gravity-stimulated to 30°, they show 420 ng/g dry weight of free IAA. In the upright pulvini, only 65 ng/g dry weight of IAA is found. The lower halves contain about 300 ng, while the upper halves contain only about 120 ng/g dry weight of IAA. We are now analysing the differences in contents of conjugated (amide or ester) and free IAA. For similar curvatures (30 degrees), the upper halves accumulate the more inactive, non-polar GAs and their conjugates while the lower halves accumulate the more active, acidic GAs. Ethylene too is released, more from the lower halves of gravity-stimulated pulvini than from the upper halves. What role ethylene plays in the gravitropic response, and if its occurrence is simply a consequence of increased GAs &/or IAA on the lower side, is not known.

The grass pulvinus offers an excellent system for studies of both the perception phase and the response of gravitropic reactions. Previous studies on gravitropism have been biased towards use of rapidly responding shoot tips, coleoptiles and root tips. But these are organs where cell divisions also take place. Also, most of these systems are responsive to phototropic stimulus. When gravitropic bending occurs, the growth response is rather diffuse over a certain length of the organ. The grass pulvinus, on the other hand, is a mature organ where cell divisions are not taking place. Growth is merely by cell elongation. The response is localized to only the small pulvinus. In most cases, the pulvini are not responsive to phototropic stimulation.

The system offers opportunities for several lines of investigation: Among them are the developmental history of an organ preparing itself to respond to gravity; the organization of a mature gravity-sensitive structure with its precise localization of tissues including the statocytes; the nature of the rapid response to auxin; the unique response of the collenchymatous cells that may lead to a better understanding of the mechanism of cell elongation; and the nature of hormonal interactions in this region. It is of significance that enclosed within the sheath pulvinus lies the stem internode, which in meristematic stages is extremely responsive to GA but not to IAA. It also responds to gravistimulation.

D12

GRAVITY RESPONSE IN THE GRASS PULVINUS IS CELLULAR ASYMMETRY THE ANSWER TO PERCEPTION?

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The gravity-sensitive grass pulvinus is a mature, non-growing organ located between gravity-insensitive, mature stem tissue below and leaf sheath above. The pulvinus responds within 20 to 50 minutes after reorientation from the vertical to the horizontal. With the help of an angular position transducer and a continuous recorder, it is possible to follow the growth kinetics over a long period of time. It is also possible to study the response of the pulvinus to treatments with hormones and inhibitors and to physical damage using this apparatus. The pulvinus itself can be halved, and in some species(wheat, corn), isolated from the rest of the segment and made to respond to gravity stimulation. The geometry of the growth response can be studied through macrophotography.

The pulvinus is very sensitive to auxins. Indole-3-acetic acid, applied in lanolin to one side of the vertically-held segment, can cause a bending response within 5 minutes. The curvature elicited by IAA can be greater than the curvature brought about by normal gravity stimulation for a comparable time. That IAA is involved in the growth response of the pulvinus is further strengthened by the following observations: Inhibitors such as triiodobenzoic acid and chloroflurenol (a morphactin) completely inhibit the gravitropic response. Brassinolide, a steroid lactone, when applied to the lower side of the horizontally-placed pulvini, causesgreater curvature than in the controls. It is known that brassinolide causes growth responses only when IAA is present in the sytem or supplied to it. Yet another piece of supporting evidence is our finding that the pulvinus alone(and not the tissue above and below) has the capacity to respond to wounding. Both the sheath and the internodal pulvini can respond by cell elongation at the wounded region. This response offers hopes for the culturing of the gravity-sensitive pulvinar tissues to study their responses in vitro.

The involvement of other plant hprmones points to the complexity of the response of tissues in this region. Gibberellic acid has no effect on vertically-held segments. But, it increases curvature considerably when applied to the lower side of horizonatally-placed segments. Kinetin and isopentenyl adenine also cause similar responses, although to a lesser extent. Abscisic acid causes a significant decrease in curvature. Pretreatment with colchicine, actinomycin D and cycloheximide also interferes with the gravitropic response.

As an approach to the study of the perception phase, we are analysing the geometry of the growth response. The epidermis, in particular, can be studied throughout the gravitropic response of the pulvinus. While the epidermis on the lower side elongates, the upper epidermis

offers some resistance to upward bending. If the upper epidermis is scratched during bending, there is a sudden increase in the rate of bending. Conversely, if the lower epidermis is damaged, there is a sudden depression in the rate of bending.

While the epidermis on the upper surface does not grow, and that on the lower surface grows maximally, the epidermal regions in between show a gradation of growth response. Thus, the usual analysis of the upper and lower halves for the determination of hormones or of other compounds is only a crude approximation of the actual situation. The geometry of the growth response in the pulvinus makes it possible to expect a fundamental distiction between the gravitropic response of unicellular organs (rhizoids, hairs, hyphae) and multicellular organs. In the multicellular organs (shoots, roots, coleoptiles, petioles), there must exist a fundamental radial asymmetry at the cellular level. This asymmetry could be of a biochemical nature, as with enzymes, hormones, or other growth-controlling or gravity-sensitive substances distributed differentially inside the cell membrane or wall. It should be possible to extend the model created for the grass pulvinus to other organs, such as coleoptiles, hypocotyls and even roots when their longer responding regions are also taken into consideration.

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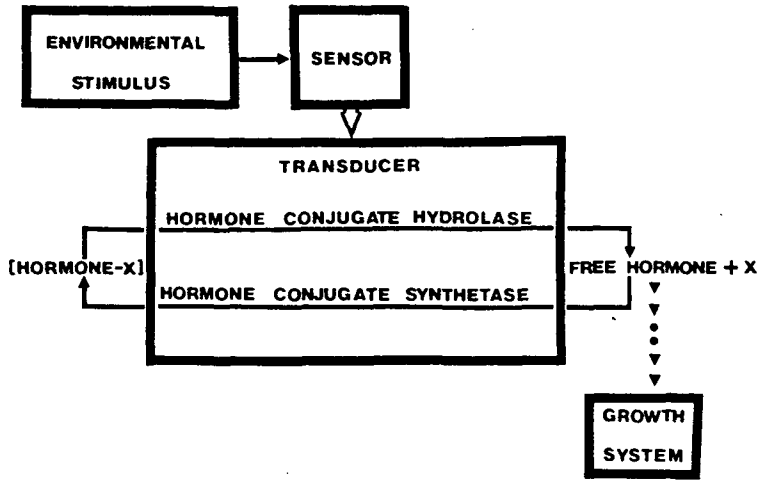
D/13

I. Introduction

All plants examined contain, as much as, 90% of the growth hormone, indole-3-acetic acid (IAA) as ester or amide conjugates and only 10% as the free, active hormone IAA (1,2). Following geo-tropic stimulation of young *Zea* and *Avena* seedlings there is a greater concentration of free IAA on the lower rapidly growing, side of the seedling. We propose, as a working hypothesis, that the assymetry in distribution of IAA is owing to differential hydrolysis and synthesis of the conjugates on the upper and lower sides of the geo-stimulated tissue.

A diagrammatic representation of this hypothesis is shown below (3):



Our research plan is to study geo-induced changes in distribution of growth hormone and its conjugates in the belief that that knowledge will lead to identification of the geo-sensing mechanism. The research planned will contribute to knowledge of the geo-growth response irrespective of the correctness of our working hypothesis. The working hypothesis proposed is testable.

II. Assay of indole-3-acetic acid:

The growth of *Zea* and *Avena* seedlings is limited by indole-3-acetic acid (IAA) and IAA is most likely the hormone involved in the geo-growth response of cereal seedlings response (cf. 4). We earlier devised ^{14}C-IAA isotope dilution assays (1,2,5) for IAA but these assays were limited to large amounts of tissue. Recently we synthesized and utilized 4,5,6,7 tetradeutero IAA as an internal standard for a gas chromatographic-selected ion monitoring-mass spectrometric (gc-sim-ms) assay which allows quantitative analysis of IAA from as little as 10 gm of tissue. This assay enabled us to confirm the geo-induced assymetric distribution of free IAA previously based only on bioassay (7) as is shown below (8):

Table 5. Amounts of free IAA and ester in the upper and lower halves of geotropically stimulated *Zea* shoots [a]

	μgm · gm dry^{-1}	pmol · 1/2 plant^{-1}
Free, upper half	0.37 ± 0.03	5.3 ± 1.3
Free, lower half	0.57 ± 0.09	7.6 ± 1.2
Ester, upper half	1.35 ± 0.34	19.2 ± 1.4
Ester, lower half	1.32 ± 0.10	18.9 ± 1.1

[a] Mean of 10 determinations using n − 1 = 9 to compute the standard error of the mean

The precision of our data was not sufficient to establish stoichiometry between ester and free IAA. Further, and disturbingly, we found that free and ester IAA occurred in two separate pools - free IAA predominantly in the vascular bundles and ester IAA predominantly in the cortex (9):

	Free IAA	Ester IAA
	(pmol·plant^{-1})	
Bundle	11 ± 1	1 ± 1
Cortex	3 ± 0.6	16 ± 4

III. Projected work:

This finding of compartmentation complicates the attempt to establish stoichiometry between free and ester IAA. We need an even more sensitive assay to enable the determination of free and ester IAA in cortical and epidermal strips and in vascular strands. We have done the first nagative ion-chemical ionization gc-sim-ms studies of IAA and hope to develop negative ion CI-gc-sim-ms as an IAA assay since negative ion methods can increase sensitivity of gc-sim-ms by 3 to 8 orders of magnitude (10).

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D14

AROMATIC BIOSYNTHESIS IN HIGHER PLANT TISSUES

Joe R. Cowles and H. W. Scheld

Objectives:

-- To develop a model system for studying lignification in higher plants

-- To characterize the model system with respect to synthesis of aromatic compounds

-- To elucidate the regulation of aromatic biosynthesis with particular emphasis upon gravitational effects

Methods:

Lignin was quantitated by a biochemical micro-assay and a microscopic assay presently under development. PAL activity was assayed using ^{14}C phenylalanine. High pressure liquid chromatography (HPLC) was carried out on a Laboratory Data Control (LDC) instrument equipped with a variable wave-length detector; the phenolic compounds were monitored at 254 nm. The column system consisted of a Whatman precolumn and a Waters μ-Bondapak C_{18} column. The elution solvent was water-acetonitrile-acetic acid.

Discussion:

Lignification in developing slash pine seedlings can be observed in walls of xylem cells within 2 days after germination. As the seedlings elongate, lignification continues upward at a rate comparable to the elongation rate. Approximately 5 days after germination lignin can be detected in epidermal cells just

above the root collar. Lignin synthesis in the epidermal cells subsequently progresses up the hypocotyl.

Lignification in developing pine seedlings was highly light-dependent. Seedlings grown in the dark contain 6- to 8-fold less lignin than light-grown seedlings. The recovery of lignification in dark-grown seedlings was affected by light intensity. Overall, the rate of lignin synthesis during the recovery period was affected by position within the hypocotyl. Hypocotyl position was less of a factor in older seedlings. Rate of lignification was inversely related to hypocotyl length during the recovery period.

Seedlings subjected to horizontal rotation (clinostating) for 10 days contained 20-25% less lignin in the hypocotyl base than did the vertically-rotated controls. Horizontal rotation also increased PAL activity (> 50%) in the basal hypocotyl region. The enhancement of PAL activity is of greater magnitude and in the opposite direction to that of lignin. This suggests that clinostating may also cause several other physiological changes. The effect, if any, of hypergravity on developing pine seedlings will be reported.

Lignin is the principal polymer synthesized via the C_6-C_3 pathway but not the only aromatic compounds. HPLC has been used to separate the major soluble aromatic compounds in developing slash pines. Four of the compounds have been positively identified using various techniques.

The initial enzyme that directs substrated into the C_6-C_3 pathway is phenylalanine ammonia-lyase (PAL). The activity of this enzyme has been shown to be correlated with the synthesis of various aromatic end-products including lignin. Experiments in which the epidermal region of the hypocotyls were removed and assayed for PAL activity showed a strong correlation between PAL activity and lignin content in the epidermal region of developing pine seedlings. Most of the PAL activity in pine hypocotyls was in the epidermal region and in the soluble fraction of that region.

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NSG 9047
Sept. 9, 1980

MAMMALIAN GRAVITY RECEPTORS: STRUCTURE AND METABOLISM

Objectives

The long term goal of my research remains elucidation of calcium metabolism in the gravity receptors of the mammalian inner ear. The immediate goals of the past year were: 1) to find a method of studying otoconial complex uptake and release of calcium ions in vitro that would permit reduction of the data to a picomoles calcium accumulated per microgram dry weight; 2) to ascertain the effect of reversal of the normally high K^+/Na^+ ratio upon calcium ion binding and release through in vitro studies; and 3) to begin the search for a suitable method for analysis of the protein composition of the organic material of the otoconial complexes.

Background

Otoconial complexes add mass to the gravity receptor organs, comprising what is called a "test mass", thereby increasing the sensitivity of the receptors to forces of linear acceleration. What is less commonly appreciated is the fact that the presence of calcium and of carbonate ions in the "test mass" of most vertebrate gravity receptors indicates that phylogenetically there was a need to store calcium ions against periods of short supply; that they were stored with carbonate ions should reflect the fact that carbon dioxide and carbonate ions were plentiful but phosphate ions, which occur with calcium in the apatite mineral of bones and teeth, were not. A further deduction is that the protein over the cells of the macular regions was chemically appropriate to capture the calcium ions and drive them into a ground energy state. Thus the ions are stored in non-active form, but can be recalled to an ionized state as required. The mechanisms by which the binding and release of the calcium ions are regulated are not known and really cannot be known until the chemical nature of the organic material in the complexes has been revealed. It is also clear that the otoconial mineral is seeded and grows in a fluid environment, and that the ions and other substances in that fluid will influence calcium ion binding and release. A high K^+ to Na^+ ratio appears to favor calcium ion sequestering in other systems, while a decline or reversal in that ratio favors calcium ion release. This would be an energy efficient way of regulating calcium ion homeostasis in endolymph, should the K^+/Na^+ ratio prove influential under controlled, in vitro conditions.

Hypotheses

1) Under in vitro conditions, the capacity of saccular and utricular otoconial complexes to bind calcium ions should be approximately similar.

2) It is expected that calcium binding will be greater when the K^+/Na^+ ratio is high and will be lower when that ratio is decreased or reversed.

3) It is expected that protein analysis of the organic material of the complexes will uncover a peptide in the molecular weight range of others (such as calmodulin and troponin-C) which are known to fix calcium ions.

Methodology

The animals used throughout have been young adult (150-400 g) Sprague-Dawley rats obtained from Charles River. Two methods have been applied for in vitro work. Method 1: Saccular and utricular complexes were microdissected under artificial endolymph (AE) or artificial perilymph (AP) prepared from data in the literature and buffered to pH 7.4 with Hepes. Complexes were incubated in 0.5 ml of AE or AP to which $^{45}Ca^{2+}$ was added to yield 1000 cpm. They were agitated in a shaker water bath (50 rpm) for 30 min, beakers covered. Complexes were then removed to a millipore filter over a Hirsch funnel for vacuum filtration, 4 pooled per sample, and were briefly washed with cold solution. Blank filters prepared similarly were used as controls. All samples were subjected to 1 N HCl (to demineralize otoconia) and prepared for liquid scintillation spectrometry. Results were expressed in cpm/patch.

Method 2: Two groups of rats were used: 150-200 g and 300 g. AE and AP were balanced ionically and were buffered by gassing with 50% CO_2. Complexes were incubated as above in a shaker water bath covered with a hood for continuous gassing with CO_2. 1.3×10^{-3}mM $^{45}CaCl_2$ had been added to the incubating mixtures, and 5,10 and 15 mins exposure times were used. Six samples were picked up on 300 mesh copper grids, dried, weighed, and prepared for liquid scintillation spectrometry. Blank grids were used as controls. Total calcium uptake was computed as picomoles/microgram dry weight.

Scanning electron microscopy of samples exposed to the incubating fluids for equivalent periods of time was carried out to observe whether or not otoconial integrity was maintained during incubation.

Protein analysis: Microdisc gel electrophoresis has been carried out on pooled samples (2-5 pairs) of otoconial complexes according to the method of Gainer (1971). Samples were denatured and solubilized in a mixture of SDS and β-mercaptoethanol prior to placing material on the gels (6 µl per gel). Standards were hemoglobin, lysozyme, β-lactoglobulin, trypsinogen, albumin.

Results

Our early in vitro results using unweighed samples showed that uptake of calcium was high in artificial endolymph but virtually lacking in artificial perilymph. Means of cpm/pa for (n) experiments were as follows: AE: saccular patches, 417±232 (19); utricular patches, 305±245 (18). AP: saccular patches, 45±69 (7); utricular patches, 29±48 (7). In AP some counts were zero. The observations fit findings obtained in other systems that sequester calcium ions, but the inability to reduce the data to uptake/unit weight was troubling and use of Hepes buffer (which added ions) was a further problem. Additionally, phosphate was present in AP but not in AE. To resolve these issues, method 2 was devised. Under ionically matched conditions, the trend for greater uptake to occur in AE was maintained but standard errors were greatly reduced. (SD pooled in AE, 150 g rats: utricular complexes, ±94; saccular complexes, ±137; in AP, utricular complexes, ±35; saccules, ±90). Uptake increased over time and was greater in the complexes obtained from young rats. For example, at 5 mins, saccular and utricular uptake was about 110 pMoles/µg in AE, for 150 g rats, and about 25 pMoles/µg in the case of 300 g rats. Scanning electron microscopic study of complexes exposed to the various incubating media demonstrated that otoconial integrity was preserved in all cases.

Protein analyses are still ongoing. Results thus far indicate that there are two major proteins, one of higher molecular weight (~60-70,000) and the other of lower weight (~16-18,000 Daltons). These molecular weights are tentatively assigned, as further work is under way to better characterize them. Several minor protein bands also are evident in the gels. The preliminary conclusion is that the organic material of the complexes at the two sites is identical.

It is the protein in the range of ~16-18,000 Daltons that is attracting our attention. This molecular weight is in the range of calmodulin and troponin-C, other calcium binding materials found intracellularly and in muscle, respectively. As the calcium-fixing segments of proteins involved in calcium regulation in other systems are relatively stable phylogenetically, it is expected that future research will reveal a highly acidic peptide in otoconial complex protein that will prove similar in amino acid composition to other calcium fixing materials already well known.

Current efforts

Currently, our research is aimed at better characterizing the proteins of the otoconial complexes. Once the proteins have been separated and we are satisfied that all have been identified, amino acid analysis of total protein content and of at least the major bands will have to be carried out. The purpose is to determine whether or not a calcium fixing protein identical to or analogous to other known proteins that bind calcium is present.

Reference: Gainer, H. 1971 Microdisc electrophoresis in sodium dodecyl sulfate. An application to the study of protein synthesis in individual identified neurons. Anal. Biochem. 44, 489-605.

RESEARCH ON PRENATAL AND POSTNATAL DEVELOPMENT AND FUNCTIONAL DIFFERENTIATION OF THE VESTIBULAR SYSTEM IN NORMAL, ROTATED, AND CENTRIFUGED RATS AND MICE

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The development of manned spaceflisht has elevated our awareness of sisnificant biolosical implications of sravitation in the normal physiolosical processes. Such sravitational effects as altered cardiovascular states and neurolosical disorientation due to unusual sensory inputs results in a hishly debilitatins syndrome durins the first several days of manned flisht. Adaptive resolution of this state is achieved by marked alterations in cardiovascular dynamics and throush the plasticity of the central nervous system to altered sensory inputs. (Berry,1975; Graybiel etal.,1977).

The analysis of potential spaceflisht effects upon developins biolosical systems has been limited in the past to Amphibian, Piscean and Avian species. Thus, Youns and coworkers studied the early developmental stases of Rana development durins the Gemini and Apollo missions, concludins that developmental stases beyond the second cleavase were NOT adversely affected by spaceflisht factors (Youns etal.,1971). Scheid and collaborators analysed the development of Fundulus embryos from mid-sastrula stases thru hatchins on ASTP and Cosmos 782 and failed to observe any effects attributable to the spaceflishts proper (Scheid etal, 1977; Keefe etal, 1978). It must be stressed that all of these studies were undertaken on normally free-floatins fertilized esss.

A Joint Soviet-American project durins Cosmos 1129 save a first opportunity to study the development of fertilized Avian esss (Coturnix). Althoush sisnificant problems with both preflisht storase (elevated temperatures/lack of rotation) and inflisht humidity control (R.H. dropped to 25%) in the incubator severely impacted this study, development of several of the esss to "normal" developmental stases provided evidence of a lack of spaceflisht effect directly upon Avian embryosenesis (Keefe, Studies in prosress).

The lack of experiments dealins with mammalian development reveals the history of manned spaceflisht to have been truly that of "manned" spaceflisht! With the sinsle exception of Cosmonaut Valentina Tereshkova (Vostok 6 - 1963), ALL Astronauts have been males. Only six other female mammals have spent appreciable times in spaceflisht! one of the "Pocketmice" (Perosnathus) flown on Apollo 17 and five Czechoslovakian Wistar-SPF rats flown on the unmanned biolosical spacecraft-Cosmos 1129 in October 1979. Durins the latter mission, the five female rats were joined with two male rats and breedins was potentially possible. The results of this experiment may be summarized as failins to establish the capability of mammalian females to carry-out the full reproductive process under spaceflisht conditions (Keefe, Studies in prosress).

KEEFE/PRE-POSTNATAL VESTIBULAR DEVELOPMENT IN RODENTS

The present study seeks to establish a baseline of functional development in the mammalian vestibular system. It is a PRIME OBJECTIVE of this study to provide a system of analysis utilizing morphological, quantitative autoradiographic and functional tests to demonstrate the normal developmental timing and interactions underlying the differentiation of the peripheral vestibular apparatus, the establishment of its central projections and integrations and to separate these within the developing CNS from other, potentially spaceflight affected, systems (such as Proprioceptive).

The study will utilize normal, rotated and centrifuged rats and mice. Furthermore, the strain of mice to be studied (C57Bl/6J) as normals have a specific mutant strain, termed the Pallid mutant (Lyon, 1955), which when used in experimental combinations involving rotation and/or centrifugation should permit us to distinguish otolithic (gravimetric) activity from canalicular (rotational) or proprioceptive inputs/pathways.

The autoradiographic techniques to be utilized in this study include the standard application of tritiated thymidine injection of pregnant females on known days of pregnancy with analysis of the autoradiographs prepared from the sacrifice of the newborn (PN28) pups establishing the times of neuron and membranous labyrinth differentiation. A second autoradiographic technique involves the functional analysis of cells by measuring their rates of glucose utilization during various activity load patterns (resting, slow continuous rotation, slow variable rotation, centrifugation). This technique is based upon the observations by Sokoloff and others that cells of an animal given an intravenous injection of 2-Deoxyglucose incorporate and accumulate this glucose analogue although the 2-Deoxyglucose-6-phosphate is unable to leave the cells. The amount of labelled DEOG in the cell body and processes of both secondary and integrative neurons has been shown to be directly correlated with the elevation of glucose utilization in appropriate CNS regions (tracts and nuclei) with increased activity of the relevant sensory inputs.

The proposed study is designed to utilize the combination of selected sensory inputs (resting vs rotation vs centrifugation) presented during sequential development from GD6 to PN28 with DEOG to demonstrate the functional differentiation and activation of the labyrinthine and gravimetric components of the vestibular system and to distinguish the differentiation of these sensory projections from other (E.G., proprioceptive, oculomotor) systems.

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D17

ABSTRACT OF PRESENTATION

FOR NASA/AIBS SYMPOSIUM ON SPACE BIOLOGY

AMES RESEARCH CENTER, 2-3 OCTOBER 1980

Avian Embryonic Development in Hyperdynamic Environments

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Two centrifuge incubators are now in use -- and instrumented for the continual monitoring of the internal environment. This has required resolving a series of technical problems, few of which were anticipated. The centrifuge incubators are simple redwood boxes with two compartments -- one for heating and humidifying and the other for incubating eggs. The heating chamber has a 250 watt heating element that is regulated by a thermistor temperature controller (YSI 63RC) -- which is mounted on the centrifuge turntable. It also has a water container, compatible with the variable water surface (resulting from zero-degrees of freedom mounting), heated by a 100 watt cartridge heater, which operates simultaneously with the air heater. A low vibration blower (Rotron *Minispiral*, SE2B21) assists humidification. Temperature is monitored by a thermistor probe (Cole-Parmer 8438-00), with the output transferred through a low-noise slip-ring assembly (Superior Carbon SK1410) to thermistor thermometer (YSI 47) that is mounted on the centrifuge control panel. The latter has 11 channels that are scanned sequentially, with the temperature recorder (YSI 80A). This allows for a continuous monitoring of temperatures and humidity in the room and in the incubators and hatchers at one or five minute intervals. Humidity is determined from the wet-bulb temperature, recorded from a (vinyl potted) rectal-esophageal thermistor probe fitted with a wet-sleeve.

Ventilation in the incubation compartment is achieved by two high capacity, low-vibration blowers (Rotron *Biscuit*, BT2A1). One draws air from the incubation compartment, with the output directed towards the heaters in the heating/humidifying compartment. The other blower stirs the air in the incubation compartment, to limit zonal temperatures.

The centrifuge hatchers (developed from existing centrifuge cages, and mounted with one degree of freedom) are essentially completed. These also are two-compartment boxes -- one containing the heating, humidifying and air circulating apparatus, and the other, hatching trays. Temperature and humidity control and recording are done the same way as with the incubators.

A series of incubation experiments have been carried out with a four-way comparison of acceleration effects: eggs laid at 1 G or 2 G, and incubated at 1 G or 4 G. Among the various abnormalities noted, several appeared rather consistently:

Eggs laid at:	2 G	2 G	1 G	1 G
Incubated at:	2 G	1 G	2 G	1 G
Number of eggs:	16	50	195	150
Infertile:	7 (44%)	23 (46%)	3 (2%)	---
Very early mortality (PD, BWE, Cystic)	---	4 (8%)	3 (2%)	3 (2%)
Break-out Normals:				
2-4 days	---	---	33 (16%)	33 (22%)
5-17 days	---	---	31 (16%)	54 (36%)
Hatch Problems:				
DIS/pip	7 (44%)	6 (12%)	12 (6%)	10 (7%)
Hatched Chicks:				
Normal	1 (6%)	8 (16%)	20 (10%)	12 (8%)
Defective	1 (6%)	1 (2%)	2 (1%)	13 (9%)
Abnormalities:				
Circulatory defects	---	6 (12%)	35 (18%)	9 (6%)
Eye defects	---	---	10 (5%)	7 (5%)
Retarded	---	---	6 (3%)	1 (0.6%)
Anatomical changes	---	---	13 (6%)	5 (3%)
Eye defects & retarded	---	---	2 (1%)	---
Eye & circulatory defects	---	---	7 (4%)	2 (1%)
Circulatory defects & retarded	---	1 (2%)	6 (3%)	1 (0.6%)
Circulatory & anatomical defects	---	1 (2%)	5 (3%)	---
Anatomical defects & retarded	---	---	2 (1%)	---
Eye, circulatory & anatomical defects	---	---	5 (3%)	---

These results indicate several major embryonic defects which were encountered more frequently in embryos developing in a 2 G field:

1. Circulatory (especially reduced yolk sac circulation and focal body hemorrhages). This category was approximately three-times as common in centrifuge as in static incubator.

2. Eye defects (reduced, especially missing or abnormally shaped eyes) two-times as frequent in centrifuge incubator.

3. Retarded development (usually disproportionate, e.g., brain retardation) eight-times as frequent in centrifuge incubator.

4. Miscellaneous anatomical defects, including twisted trunk, limb anomalies, and celosomia were four-times as common in the centrifuge incubator.

Several of these defects also result from high incubation temperatures and these were also more frequent than would be expected in the statically incubated eggs -- so at present, there is some uncertainty as to the contribution of non-acceleration environmental effects in their occurrence. However, the current instrumentation, permitting precise control and continuous recording of the development environment will allow us to delineate acceleration effects.

D18

Establishment of the dorsal/ventral polarity of the amphibian egg-- use of egg rotation re. gravity and ultraviolet irradiation as probes

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Several hypotheses were tested concerning the nature of the cytoplasmic rearrangements which accompany activation of the amphibian egg and the establishment of its dorsal/ventral polarity. The results of a variety of experiments are summarized under the following headings.

I. Egg rotation re. gravity and UV as probes: UV irradiation of the uncleaved egg was discovered to alter the natural position of the dorsal lip at the gastrulation stage. Irradiated embryos appeared to have lost their capacity for normal symmetrization. The dorsal lip, for example, frequently formed on the same side on which the fertilizing sperm had entered.

A brief rotation re. gravity of the uncleaved amphibian egg succeeded in relocating the dorsal lip to a novel site in the embryo. That site almost always opposed gravity during the rotation, and even coincided with the sperm entry side in some embryos.

Those brief rotations of irradiated eggs were capable of preventing the development of the abnormal neural morphologies associated with embryogenesis of UV'd eggs.

II. Responses of anuran vs. urodele amphibian eggs to rotation and irradiation probes: A variety of anuran and urodele amphibian eggs were studied with the rotation and UV probes. In all instances, the dorsal lip formed on the side of the embryo which had opposed gravity during the brief rotation of the uncleaved egg. Anuran and urodele eggs responded to the combined use of rotation and irradiation in a similar fashion. Despite the superficial differences in the fertilization process of anuran and urodele eggs, their cytoplasmic rearrangements during activation are apparently similar.

III. Involution sites at novel locations on the amphibian embryo: Both anuran (Rana) and urodele (Ambystoma) amphibian eggs were subjected to prolonged unnatural rotations re. gravity. In some instances eggs were rotated 90°, while in other instances eggs were rotated 180° (complete inversion). Alterations in the pigmentation pattern, cleavage pattern, and site of involution were observed. Despite those unnatural orientations re. gravity, morphogenesis was normal in many cases.

The above findings have been used to generate a model for the establishment of the dorsal/ventral polarity of the egg. Rather than being localized in a fixed position in the egg (eg. the egg cortex), the determinants for the pattern of early embryogenesis are probably located in that portion of the internal cytoplasm which responds to gravity orientation. Data from the UV studies indicates that a surface component is, however, probably required for normal egg orientation.

D9

A Ground-Based Comparative Study of the Effect of Weightlessness on the Development of Amphibian Eggs

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The establishment of bilateral symmetry and the development of gametes (eggs and sperm) are two phenomena which may be influenced by the normal gravitational field. Two questions may be asked. Can bilateralization take place in the absence of gravity? Can eggs or sperm develop in the gonads of vertebrates which do develop in the absence of gravity? Hypotheses are available, but definitive data are lacking.

Bilateralization: The vertebrate egg is biradially symmetrical - like a pear. After fertilization, the egg membranes elevate thus forming a fluid filled space. Because of this the fertilized egg, whose hemispheres have different densities, becomes free to rotate in the gravitational field. Within minutes, and before the first cleavage division, the zygote can be recognized as bilaterally symmetrical. Tumbling or rotation of the egg in the tubes or during egg laying before fertilization does not accomplish this. Activation of the egg is necessary.

The favored hypothesis suggests that the plane of bilaterality is coordinated with the plane of rotation. Another suggests a relationship between the plane defined by the axis of the egg and the point of sperm entry and the plane of bilaterality. This may be a "back-up" system. Restriction of rotation, centrifugation, or clinostat exposure (gravity compensation) can result in the production of abnormal embryos.

Gamete specification: Germinal granules define cells destined to become gametes. Before fertilization these granules are widely distributed in the cytoplasm but they become localized near the vegetal pole at the same time bilaterality is being established. How these granules become localized or how they influence gamete formation is not known. The timing suggests their localization is related to the same events that determine bilaterality. Current hypotheses are poorly formulated on this topic - observations are needed.

Current work: Flight studies are needed to test these hypotheses as the manipulations involved in ground-based studies introduce variables which can affect the results. Prior to orbital flight (see Proposal No. 781256 - Tremor et al.), (1) suitable animals must be obtained, maintained and tested, (2) comparative clinostat studies must be conducted, and (3) criteria for evaluation must be defined.

Orbital flights are not coordinated with the reproductive cycles of frogs. However, the Amphibian Facility of the University of Michigan permits full year availability of gravid animals and the conduct of necessary ground-based studies.

Using Rana pipiens, the leopard frog, in gravity compensation studies, Tremor and Sousa demonstrated that at specific clinostat rotation rates developmental abnormalities are produced. Were these results peculiar to R. pipiens? We (including Karen Metzger and Aaron Mendel) are extending them to Xenopus laevis, the African clawed "toad", and Bombina orientalis, the oriental fire-bellied "toad", each representing different genera.

Our initial effort is designed to define the specifics of egg rotation. Photographs of rotating eggs taken at intervals are submitted to digitizer analysis. This analysis yields information on the size of each egg and its orientation at each interval. After the first rotation is completed the egg-jelly masses are inverted and the measurements are repeated. This is done twice. The data are processed to yield the orientation angle and rotation rate at each interval.

Preliminary results suggest that egg rotation starts slowly, reaches a maximum rate, then slows as the definitive position is approached. Rotation rates and dynamics for the first rotation differ from those for "artificial" second or subsequent rotations. There are differences between eggs in a clutch and between eggs from different females and species. The reasons for these differences are under examination. Clinostat studies (rotation rates, duration) will be guided by the results of these descriptive studies.

D20

DROSOPHILA BIOLOGY IN SPACE

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HYPOTHESIS: Our previous research on Cosmos - flown fruit flies suggests that exposure to weightlessness may not be very detrimental to the processes of cell division and differentiation responsible for Drosophila morphogenesis. However, it is reasonable to expect that altered gravity will result in important changes in the neuromuscular structure and function of this insect. Drosophila, like any flying animal, has elaborate sense organs for orientation in three dimensional space and a powerful wing musculature designed to counteract the force of gravity. Since the sense organs and the muscle are involved in mating behavior, we expect that weightlessness-induced disorientation and disuse muscle atrophy will result in a striking decrease in reproductive competence. This postulated interference with the physiological and behavioral processes related to flight and reproduction is likely to compromise the survival ability of flies maintained in a space laboratory during various succesive generations.

OBJECTIVES: The long-range goal of this work is to investigate the effects of space weightlessness on the biological processes of Drosophila. Special attention will be paid to the influence of zero-g on mating, fertility, embryonic development and aging. This research is in agreement with the "Life Sciences Laboratory Development Plan Concept Verification Testing (CVT)" of June 1972, which defines as the basic goal of space biology the understanding of the role of gravity in life processes and the capability of living organisms to adapt to gravitational changes, including the effects on aging. Until flight experiments can be performed, we propose to focus on the study of the fundamental mechanisms of development and aging in fruit flies maintained under normal laboratory conditions in comparison with flies housed in clinostats or centrifuges during various periods of their life cycle. It is hoped that the proposed research will provide data of interest for interpretation of the results on Drosophila development and aging obtained in our collaborative study of flies exposed to near zero-g onboard two USSR biosatellites. Moreover, this baseline work may provide useful information for the planning of Shuttle Drosophila experiments.

METHODOLOGY: The methods used in order to achieve the above goals include the following: 1) Determination of physiological competence of the flies as shown by negative geotaxis, mating, fertility and oxygen consumption. 2) Study of age-related cytological and histological changes by routine light microscopy. 3) Investigation of developmental and degenerative changes in mitochondria using the methods of electron microscopy morphometrics. 4) Determination of the glycogen content of the wing muscle. 5) Biochemical analysis of the key enzymes concerned with the energy metabolism of the flight muscle, such as Ca-activated actomyosin, ATPase and arginine phosphokinase.

RESULTS: 1) We have shown in collaborative studies with Dr. Morris Rockstein, from the School of Medicine of Miami, that the key enzyme of energy production in the flight muscle of Drosophila starts declining at 4 to 5 days after emergence of the adult flies. This finding is in agreement with previous studies from our laboratory showing that neuromuscular competence (as shown in mating and negative geotaxis) starts its decline at about the same age. The ATPase technique is a new addition to the battery of techniques to be used in future flight experiments to investigate the effects of exposure to zero-g on muscle biochemistry and function, both in flies and mice.

2) We have finished a comparative study of mechanisms of aging in insects (as shown by fruit flies) and mammals (as exemplified by the laboratory mouse of the C57BL/6J strain). Our data show that the non-dividing cells of both insects and mammals experience similar age-related structural changes. The most significant of these are occasional rod-like inclusions in the nuclei and a number of cytoplasmic alterations, such as the accumulation of age pigment containing oxidized fat and a decrease in the number of structures responsible for energy production (mitochondria) and protein synthesis (ribosomes). There is also evidence of a degradation of mitochondria resulting in the formation of inert membranous material. The fundamental differences between mammalian and insect aging are as follows: a) In contrast to old flies, which show pervasive senescent changes in all of their tissues, old mice show little or no evidence of age-related structural alterations in several of their tissues, such as the intestine, which regenerate through the processes of growth and division of non-differentiated cells. b) Old flies do not show structural changes in their extracellular collagen. Conversely, collagen is very abundant around certain cell types and blood vessels in the tissues of old mice. Our finding of an age-related disorganization of mitochondria in the tissues of normal old flies, with formation of peroxidized lipid debris, supports our working hypothesis that an increase in oxygen consumption because of stress-associated increase in activity was responsible for the short life span and increased amounts of lipid-containing pigment in the Cosmos-flown Drosophila.

3) We have investigated the effects of the intercalating dye ethidium bromide (EB) in order to obtain information on the role of mitochondrial respiration on development and aging of Drosophila. Ethidium bromide (EB) at a concentration of 2×10^{-4} M in the medium increased the development time of Drosophila melanogaster by 32%. Concentrations of 2×10^{-5} had no effect on development and flies failed to develop in media containing 2×10^{-3}. The adult body weights of the flies which developed on the EB-medium were 88% of the controls for the males and 77% of the controls for the females. Both the mean and the maximum life span of the flies which developed in medium containing EB were increased by 21.5% and 17.9%, respectively, when fed control food as adults. Futhermore, flies allowed to develop on control food had both mean and maximum life span increased by 22.6% and 30.2%, respectively, when fed 2×10^{-4} EB as adults. The oxygem consumption

was lower for both flies treated with EB during their development or during their adult life. The results suggest that EB, which has been shown to produce respiratory mutants in yeast, has a similar disorganizing effect on the respiratory apparatus of Drosophila. The fact that this mitochondria-induced lowering of respiration results in increased life span supports the rate-of-living theory of aging and Harman's concept that senescent disorganization is linked with the release of free radicals in the mitochondria during the course of normal aerobic metabolism.

D21

THE VISUALIZING OF EQUILIBRIUM RECEPTOR AFFERENTS AND INTERNEURONS BY HRP AND COBALT CHLORIDE HISTOCHEMISTRY (NASA GRANT NSG 7435)

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Introduction

Two giant interneurons in each ventral nerve cord connective of the burrowing cockroach Arenivaga sp. relay gravity orientation information to the brain (Walthall and Hartman, 1981). The equilibrium receptors driving these interneurons are singly innervated pendulous sensilla arranged in two rows on the ventral surface of the cerci (Hartman et al., 1979). In adult male insects, these sensilla, termed tricholiths, number eight in the lateral row, and nine in the medial row. The tricholiths in the lateral rows signal pitch forward and roll while those of the medial rows signal roll and pitch backward. In contrast to adult males, newborn insects have but one equilibrium receptor on each cercus, adding a pair at each molt (Hartman and Bennett, 1981).

Hypothesis

An equilibrium system consisting of only two gravity receptors and four interneurons in a newborn animal which grows and matures by the addition of pairs of gravity receptors at each molt is suitable for the study of the long and short time effects of equilibrium sensory deprivation on the morphology and function of sensory neurons and interneurons.

Methodology and Results

Our efforts for the past twelve months have been directed toward devising histochemical techniques which will allow us to visualize the gravity receptor neuron terminals and the dendritic branches of the interneurons in the terminal ganglion of normal adult male and female Arenivaga sp.

By backfilling the cercal nerve with horseradish peroxidase (HRP), developing the reaction product, and clearing the cerci with methyl salicylate, we have determined the innervation pattern of the gravity receptors of the cerci. The tricholiths are innervated by single afferents, the bipolar cell bodies measuring 9μ x 15μ. The axons are about 1μ in diameter. The axons of each row form discrete subnerves which are bilaterally symmetrical about a line running between the two rows of equilibrium receptors.

In order to trace the pathway of the afferents into the neuropil of the terminal ganglion and to map the terminal arborizations, the enzyme HRP must be taken up by the dendrites and transported throughout the cells by axoplasmic flow before histochemical visualization of the enzyme.

Histochemical visualization requires fixing the ganglion, then incubating it in a medium containing H_2O_2 and a chromogenic aromatic amine. The chromogen polymerizes and assumes an intense color (usually blue or brown) upon oxidation and allows visualization of the neuron. While the reaction product obtained with the 3,3' diaminobenzidine (DAB) chromogen allows identification of larger neurons and processes, the chromogen benzidine dihydrochloride (BDHC) permits visualization of the bipolar receptors in the cerci. However, the latter is not satisfactory for detecting the finest processes of axon terminals in the neuropil. Further, DAB and BDHC share the considerable disadvantage of being carcinogens for humans (Hadidian et al, 1968). We have recently modified the method of Mesulam (1978) which employs tetramethyl benzidine (TMB), a "presumptively non-carcinogenic chromogen", to our needs. This procedure provides superior morphology and sensitivity.

The giant interneurons are being mapped using cobalt chloride histochemical techniques (Pitman et al., 1972). Backfills have yielded excellent fillings of axons and cell bodies. But, in order to resolve the finest processes of the dendrites in the neuropil, a modified Timm's intensification procedure has been adopted (Bacon and Altman, 1977). We have just begun experiments to identify by intracellular injection of $CoCl_2$ the two neurons in each connective which relay gravity orientation information to the brain.

We are at the threshold of routinizing the histochemical methods described. Once the morphology and connectivity of the afferents and interneurons has been realized, the analysis of these neurons in animals deprived of gravity receptor input will follow.

D22

THERMOREGULATION IN RATS: EFFECTS OF VARYING DURATION OF HYPERGRAVIC FIELDS

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HYPOTHESIS Experiments were centered on the effects of hypergravitational fields on the thermoregulatory system of the rat. The ability of the animal to regulate core temperature when exposed to a one hour period of cold concurrently with hypergravic fields was determined. The central question underlying experiments completed this year was whether the response of the rat to the one hour cold exposure depends only upon the amplitude of the hypergravic field during the period of cold exposure or whether the response is also dependent on the amplitude and duration of the hypergravic field prior to cold exposure. That is, the hypothesis tested was whether the "past history" of the animal serves as a major determinant in the response of the thermoregulatory system.

OBJECTIVES The specific physiological questions considered during the current year in examining the effects of hypergravity were as follows:

1. Does the thermoregulatory response observed upon a one hour cold exposure during the last hour of exposure to hypergravity vary with the duration of the applied field (the field being applied for 4,7,13,19,25 and 37 hours)?
2. During exposure to a hypergravic field, does the response of the animal to the one hour cold exposure depend on the pattern (amplitude and duration) of the hypergravic field prior to the cold exposure?
3. During the first hour of exposure to hypergravity, what is the effect of cold? That is, what is the response to cold at the initiation of the 1.5 - 4G exposure?

Experiments resolving these questions (the specific aims of NASA grant NGS 2234 for the current year) have been completed and are outlined below.

METHODOLOGY Male, Long-Evans hooded rats were provided with Simonsen's White diet and water ad libitum, and were maintained at an environmental temperature of 22-24°C and at a 12:12 hr light:dark cycle. Thermistors (Veco 32A12, 2000 ohms at 23°C) were surgically implanted in the area of the carotid artery in each rat; in addition, some rats had thermistor implants in the hypothalamus and interscapular brown fat tissue. Lead wires from thermistors were soldered to an ITT Cannon connector MDL-9SL1. The connector was then cemented to the skull and held in place with stabilizing screws. The rats were allowed to recover from implantation for four days prior to centrifugation on a 1.34 m radius centrifuge. The unrestrained rats maintained a normal posture during the acceleration period (the gravitational field was oriented in the $+G_x$ direction since the centrifuge cages had one degree of freedom and could swing outward as the angular velocity increased). Temperature changes were recorded using a Houston 3000 Omnigraph penwriter and associated circuits. To lower ambient temperature, ice was placed in a container jacketing an inner 4 x 8 inch chamber housing the rat. The rat was free to move about this inner chamber, the only restraining factor being the cable connected to the connector on the rat's head.

RESULTS AND DISCUSSION One hour of cold exposure applied over the last hour of either a 1,4,7,13,19,25 or 37 hr period of 3G evoked a decrease in core temperature (T_c) of about 3°C. This fall in T_c was significantly greater than changes in T_c in cold-exposed rats at 1G. No significant differences were found between the measured decreases in T_c observed for the 1 hr cold exposures during the first 37 hr at 3G (Table 1). However, when rats were subjected concurrently to cold and acceleration following 8 days at 3G, they exhibited a smaller fall in T_c,

suggesting partial recovery of the acceleration-induced impairment of temperature regulation. In another series of experiments, the gravitational field profile was changed in amplitude in 3 different ways during the 3 hr period preceding the 1 hr cold exposure at 3G. (The first profile involved a step increase to 4G for 1 hr followed by 2 hr at 3G and then the concurrent 3G-cold exposure for 1 hr. The second profile involved a 0.5G incremental increase every 0.5 hr until 3G was reach (at 1.5 hr). This 3G field was maintained throughout the 1 hr cold exposure which began after 3 hours on the centrifuge. The third profile used was a step increase to 3G for 3 hr followed by a 1 hr concurrent 3G-cold exposure period.) Despite the different gravitational field profiles prior to cold, the magnitude of the fall in T_c over the 1 hr period of cold exposure was the same in all cases. These results suggest that the thermoregulatory impairment has a rapid onset, is a manifestation of an ongoing effect of hypergravity, and is not dependent upon the prior G profile. The inability of the rats to maintain T_c when cold exposed may be transient as indicated by the partial recovery of regulation by the 8th day.

TABLE 1. Effect of cold exposure on core temperature of rats at various times during centrifugation at 3G

	TIME AT 3G PRIOR TO COLD EXPOSURE							
	0 hr	3 hr	6 hr	12 hr	18 hr	24 hr	36 hr	8 days
ΔT_{20}	-1.79 ±0.18 (3)	-1.10 ± 0.31 (5)	-1.40 ±0.15 (4)	-1.64 ±0.49 (4)	-1.68 ±0.16 (5)	-1.63 ±0.35 (3)	-1.74 ±0.37 (4)	-0.45 ±0.16 (4)
ΔT_{40}	-2.54 ±0.16 (3)	-2.04 ±0.30 (5)	-2.22 ±0.20 (4)	-2.88 ±0.93 (4)	-2.48 ±0.22 (4)	-3.13 ±0.64 (3)	-2.79 ±0.54 (4)	-0.86 ±0.16 (4)
ΔT_{60}	-2.76 ±0.20 (3)	-2.53 ±0.27 (5)	-2.71 ±0.18 (4)	-2.98 ±1.70 (4)	-3.30 ±0.25 (3)	-3.90 ±0.85 (3)	-2.83 ±0.46 (3)	-1.12 ±0.20 (4)

Values represent $\bar{X} \pm$ S.E. for the number of trials shown in ( ). Values for ΔT_c are in °C. ΔT_{20} denotes the fall in T_c occurring over the first 20 min of cold exposure at 3G, ΔT_{40} the fall in T_c after the first 40 min, and ΔT_{60} the fall in T_c after 60 min.

The experiments, interpreted as indicating that the response of the rat to cold exposure is primarily dependent on the amplitude of the hypergravic field during the period of cold exposure rather than the prior amplitude and duration of the hypergravic field, are described in more detail in the following manuscripts:

1. J. M. Horowitz, E. R. Schertel and B. A. Horwitz. Centrifuge high-g effects on temperature regulation in unanesthetized rats. The Physiologist 22:S-57-58, 1979.
2. E. R. Schertel, J. M. Horowitz, and B. A. Horwitz. Effects of gravitational profiles on the rat's thermoregulatory response to cold. J. Appl. Physiol.: Respirat. Exercise Physiol. in press.

D23

HYPER-GRAVITATIONAL EFFECTS ON METABOLISM AND THERMOREGULATION

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OBJECTIVES: To determine the effects of acute and long-duration hyper-G exposures on thermoregulatory and blood glucose homeostatic functions of laboratory animals and to elucidate their regulatory mechanisms.

HYPOTHESES: Re-entry and return-to-earth after prolonged space flight exposures to weightlessness subject animals to hyper-G forces which can be stressful resulting in significant changes in thermoregulation and of functions involved with blood glucose regulation. Through ground-based centrifuge studies, these changes can be simulated providing an extremely useful technique for investigating post-flight stress effects on animal systems. We further hypothesize that important and relevant information pertaining to the effects of long-term space flight weightlessness can be obtained from investigations of adaptational changes of animals exposed to prolonged hyper-G conditions as well as to reduced-G (de-acceleration).

METHODOLOGY: Thermoregulatory responses of rats during acute hyper-G stress exposures are being monitored continuously using rectal and tail probes with a recently developed automated system for use on our 4 ft. radius centrifuge. As many as 16 rats can be monitored simultaneously during a centrifuge test run with the data collected on a magnetic tape recording system and analyzed by a specially developed program. Metabolic heat production changes during hyper-G stress exposures are being monitored through a specially designed respiratory exchange system which is also capable of measuring oxygen consumption and CO_2 production rates continuously of individual rats for periods up to 24 hours while being centrifuged. Various approches are being used to delineate key factors which can attenuate the rapid and marked hypothermic response which is characteristic to hyper-G stress in laboratory animals. Central and peripheral control mechanisms are being investigated through regulation of ambient temperatures to which rats are subjected during hyper-G stress exposures; repeated exposures to hyper-G; and pre-administration of various drugs including amphetamine and isoproterenol (beta-adrenergic agonist drug). Hyper-G exposure effects on blood glucose homeostasis are being determined through intact animal studies as well as through isolated tissue preparations. Emphasis is currently on *in vivo* gluconeogenesis rates of rats from C-14 labelled lactate, glycerol, and alanine as affected by exposure duration to hyper-G. Plasma precursor concentration changes as well as various hormones (catecholamines, insulin, glucagon, corticosterone, and thyroid hormones) affecting glucose mobilization and metabolism are being measured also as a function of exposure time.

RESULTS AND CURRENT EFFORTS: The hypothermic response induced by re-exposing previously chronically centrifuged rats (8 months) and subsequently returned to 1G for 3 months to hyper-G was indistinguishable from the response of control rats which had never been exposed to hyper-G. Studies are in progress to determine the minimum time for re-adaptation to 1G of hyper-G adapted rats using the hypothermic response as an index. Previous studies have shown that hyper-G adapted rats have a sig-

nificantly higher resting metabolic rate than normal gravity control rats. Recently completed body compositional analyses of 10-52 week old hyper-G adapted rats have shown the older rats to have a higher percentage of "active mass" than control rats of comparable body mass. These differences, however, cannot fully account for the higher metabolic rates of hyper-G adapted rats which is undoubtedly due to a generalized increase in cellular metabolism. In vivo gluconeogenesis rate changes are currently being studied in rats exposed to hyper-G beyond 1 hour duration. Plasma catecholamine levels have been found to increase above control levels after 24 hours of centrifugation exhibiting a bi-phasic pattern similar to the plasma glucose and plasma insulin responses observed earlier. Plasma glycerol measurements so far have revealed no significant differences from control levels for hyper-G exposures of 1 hour or less. This is somewhat surprising in view of the marked increase in plasma free fatty acid levels which occur during the same time period. More extended exposure periods are being investigated to see how they affect plasma glycerol levels and their relationship to glycerol gluconeogenesis rate changes. Studies are being conducted to further establish the most reliable conditions for measuring gluconeogenesis changes in hyper-G exposed rats.

D24

NASA-AIBS: Symposium On Space Biology
Ames Research Center, October 2-3, 1980.

Gravity and body composition in the rat.

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ABSTRACT

Three aspects of work during the past year are presented:

1. Final results of the Cosmos 1129 study (K-316) - "Weightlessness and body composition in the rat."

2. Comparison of Cosmos results with centrifugation results.

3. Methodology studies.

Cosmos 1129 included 5 male rats dedicated to K-316. These were sacrificed 32-36 hrs after recovery, dissected into 3 major compartments (musculo-skeletal system, skin, and pooled visera) and compared with 5 Synchronous Controls on the ground in flight-type hardware. In this comparison the Flight Group showed: a 6.7% reduction in total body water probably attributable to a 36.2% reduction in the extracellular compartment, reductions of 6.6% in musculo-skeletal water and 17.2% in skin water, an apparent shift of some water from skin to visera, and a 20% reduction in bone mineral mass. Among organ fresh masses there was a 7.5% increase in kidneys and a 14.0% decrease in spleen.

The process of comparing Cosmos results (0G-1G) with those obtained by chronic centrifugation (1G-4.5G) continues. In essence the results suggest that many of the changes observed in the group exposed to weightlessness have physiological significance even where statistical significance was not achieved.

Several methodology studies were made, one of the most important being an evaluation of the water content of the fat-free skinned, eviscerated carcass as an index to mass of muscle present. This has promise as a time-saving chemical method for "dissecting" muscle from bone.

D25

ABSTRACT OF PRESENTATION
FOR AIBS/NASA SPACE BIOLOGY SYMPOSIUM
2-3 OCTOBER 1980
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Gravitational Scale Effects

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The allometric relationship of metabolic rate (MR) to total body mass (TBM) among mature animals of the mammalian series, mouse to elephant, is widely accepted as being describable by the Kleiber/Brody equation:

$$\text{MR, kcal/hr} = 2.92\ (\text{TBM, kg})^{0.75}.$$

This equation, when logarized, yields a straight line the slope of which represents the parametric value of the exponent in the allometric equation:

$$\log\ (\text{MR, kcal/hr}) = 0.4654 + 0.75\ (\log\ \text{TBM, kg}).$$

Kleiber has postulated that the 3/4 power relationship of metabolic rate to total body mass may represent an evolutionary compromise between the 1/2 power of body mass constraint on heat transfer through the body surface dictated by Fourier's Law of Heat Flow, and the 1st power of body mass requirement for energy production to counteract the loading of the organism by gravity.

We propose to test Kleiber's hypothesis experimentally by examining the effect of changes in gravitational loading on the metabolic scale relationship in a series of mammalian species of differing body size. In the weightlessness of space flight the scaling of metabolic rate should shift from the 3/4 power toward the 1/2 power of body mass. On the other hand, if gravitational loading is augmented, as by chronic centrifugation, the scaling should shift from the 3/4 power toward the 1st power of body mass.

However, in order to carry out such experiments meaningfully, it has been necessary to identify a suitable series of mammals of size range sufficient to define scaling parameters of the allometric equation with precision adequate to detect the expected changes, yet of small enough size to be compatible with spacecraft or centrifuge dimensions. It was also important to assess the effects of age and sex difference within species so as to arrive at an optimal experimental animal model.

We started with the 5 species of common laboratory mammals: the mouse, hamster, rat, guinea pig and rabbit. This species series ranges from 0.05 kg to 5 kg mature body size, providing 2 orders of magnitude of body mass range.

Commercial suppliers provided 6 animals of a particular species, sex and age at weekly intervals. Eight age cohorts ranging from 1 month to 2 years were examined for each sex and species, making a total of 80 groups of 6 animals each to form the study matrix. Respiratory gas exchange was

determined for each animal under standard conditions as a measure of metabolic rate. We chose to employ metabolic intensity, defined as kilocalories of metabolic heat produced per hour per kilogram of total body mass, as the most convenient unit of comparison among the various groups of animals studied.

A general pattern emerged for all 5 species which indicates that metabolic intensity is very high in young animals and decreases dramatically as the animals become older until an age of 6 to 8 months is attained. At this point, the metabolic intensity stabilizes while both metabolic rate and body mass continue to increase with age. Thus, metabolic maturity does not seem to be reached until 6 to 8 months of age. Also, there appears to be little or no consistent difference in metabolic intensity between sexes within each species.

From these results it was evident that younger animals are in a highly transitory metabolic state, making interspecies comparisons difficult. In particular, the examination of scaling of metabolic rate on body size among these species would seem to be best carried out in animals of at least 8 months of age.

When the allometric relationship for the 5-species groups 8 to 24 months of age was determined, it was found to be

$$\text{MR, kcal/hr} = 3.05\ (\text{TBM, kg})^{0.676}$$

the power function of which was significantly different from the classic Kleiber/Brody equation. However, it was noted that the metabolic rate values for the strain of mice used were consistently higher with respect to the other 4 species studied. Evidence exists that different species of mice can depart substantially from predictions by the 3/4 power relationship, so that mice as a group are probably not good candidates for inclusion in a limited species series where their weighting becomes appreciable.

Accordingly, the allometric relationship for all the mature animals of the 4 species, exclusive of the mouse, was determined and found to be

$$\text{MR, kcal/hr} = 2.99\ (\text{TBM, kg})^{0.749},$$

the parameters being statistically indistinguishable from those of the Kleiber/Brody equation.

As a final test, the allometric relationship derived from the 6 male animals, 8 months of age, of each of the 4 species was found to be

$$\text{MR, kcal/hr} = 3.06\ (\text{TBM, kg})^{0.741}$$

with an accuracy of $\pm$ 5% for the scaling parameters. Closely similar results were found for the comparable 24 female animals of the same age.

It may be concluded that the 4-species series can be used to define the relationship between metabolic rate and total body mass in an acceptable and useful fashion. Therefore, it is proposed that 6 male animals, 8 months of age, of each of the 4 species, hamster, rat, guinea pig and rabbit, can be used experimentally to study scale effects of alteration of the magnitude of gravitational loading on the metabolic energy requirements of terrestrial mammals.

D26

STRUCTURAL DEVELOPMENT AND GRAVITY

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HYPOTHESIS: Skeletal growth is related to gravity and/or mechanical loading. Removal of gravity and/or loading from the weight-bearing limbs will cause a loss of skeletal mass.

OBJECTIVES: 1) To further study a model system which simulates many effects of space flight in small animals. 2) To analyze bone specimens from the latest Cosmos biological satellite, 1129.

METHODOLOGY: The basic methods for the model system have been described. The major modification in progress is the use of frictionless bearings to allow the animal access to the entire grid area rather than access limited to a circle. Experiments measuring longitudinal growth, trabecular bone mass, marrow fat, and bone cell numbers in proximal tibial metaphysis after 15-17 days of suspension have been done. Longitudinal growth in the proximal humerus was also measured. To measure longitudinal growth, rats were injected with demeclocycline, 10 mg/kg body weight, 2 days prior to termination of the experiment. The tetracycline served as a marker so that the distance of the tetracycline fluorescent band from the growth plate-metaphyseal interface could be used to estimate longitudinal growth during that 48 hours. Similar methods were used to assess the Cosmos bone specimens except that no tetracycline could be given 48 hours prior to the termination of flight in the unmanned Cosmos spacecraft. The fractional area of trabecular bone and the fractional area of fat in the bone marrow were quantified in a 2 by 2.5 mm area of the proximal tibial metaphysis with the aid of a Merz grid. The Merz grid was also used to quantify the rate of periosteal bone formation in the tibial and humeral diaphyses of Cosmos rats.

RESULTS: In rats suspended for 15-17 days, a significant decreased in longitudinal growth occurred in both the tibia and humerus as compared to pair-fed controls (tibia--95$\pm$38.2 vs 250$\pm$49.1; humerus--60$\pm$25.1 vs 201$\pm$30.8; mean in units of μ/2 days $\pm$ 1SD). Although these values are significantly different, measurement of total bone length would not show differences since growth at this rate over 20 days would only be about a difference of 1.4 mm between groups which is in the range of biological variability as well as the sensitivity of the calipers. In these rats, the first 250μm below the growth plate (where bone volume is similar) contained fewer osteoblasts in the harnessed animals as compared with controls (21.0$\pm$3.3 osteoblasts/mm bone surface vs 27.5$\pm$2.5) and more osteoclasts (4.3$\pm$1.0 vs 1.7$\pm$0.3). An increase in cell number does not necessarily reflect a change in cell activity. These rats also exhibited similar changes in % trabecular bone mass and % marrow fat as did rats from Cosmos 1129; bone mass decreased whereas marrow fat increased.

The Cosmos 1129 data confirmed earlier findings and produced additional data. Periosteal bone formation rate decreased significantly in not only the tibia but also the humerus as compared with either flight or vivarium controls. The decrement in humeral formation was not as extensive as in the tibia and may be due to the lower rate of bone formation in the humerus. A rebound in tibial formation was noted postflight as compared with either control; the humerus did not exhibit a similar rebound but periosteal bone formation did return to normal during the postflight period. No significant differences were found among the groups in cross-sectional medullary in the tibial or humeral diaphyses which implies no gross change in endosteal bone resorption occurred during the experiment. Stable calcium isotope studies showed that bone resorption decreased about 25% during flight beginning around day 12. A decreased trabecular bone volume and an increased fat content of the bone marrow in the proximal tibial metaphysis appear to be a consequence of spaceflight. The bone marrow fat content appears to return to normal within 29 days of flight whereas the trabecular bone volume remains depressed. Verification of the trabecular changes were found in vertebral strength and trabecular spacing and architecture alterations which also remained abnormal.

No significant change in periosteal bone formation was found in the rib. However, similar data were produced on the mandible using tetracycline labeling. When the mandibular tissue was subjected to density gradient centrifugation, a significant increase in the fraction of immature bone was found. Similar studies are in progress for the rib.

Measurement of the nuclear volume of osteoprogenitor populations in the periodontal ligament of Cosmos 1129 rats, suggests that the progenitor cell population was normal but that the preostoblast population was decreased during flight. The time required for the progenitor cells to differentiate into preosteoblasts is about 3 days, which is the time calculated for periosteal bone formation to begin following flight.

CURRENT EFFORTS: Experiments in which animals are suspended for 3, 5, or 7 days have been completed. Analyses are in progress to define the time at which the bone changes are first detectable. Additional analyses on the Cosmos specimens are in progress.

D27

Abstract: AIBS/NASA Space Biology Symposium

October 3, 1980

NASA Ames Research Center

Title: Hypokinesis and Antiorthostasis; An Animal Model for Studies of Problems Involved in Weightlessness

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There is growing evidence that hypokinetic and antiorthostatic responses can be achieved using our suspended rat model. Sprague Dawley, male subjects have been used exclusively. During the current year research projects have focused on (1) extending the period of hypokinetic suspension from seven to fourteen days to determine the feasibility of longer periods of suspension and to evaluate the nature of responses which result from two weeks of antiorthostatic hypokinesia; (2) repetition of earlier projects (in a new laboratory with new personnel) in order to assess the reproducibility of previous research programs and, (3) to examine aspects of food and water intake in the physiological modification which resulted from suspension. The latter area of investigation was in great part the result of questions raised during previous reviews.

(1) Suspension of a rat such that the animal is permitted use of the forelimbs but with removal of weight bearing function of the hind limbs has been readily achieved for a one week period and is now being utilized for two week tests. During a two week suspension the rats respond with typical: decline in growth, diuresis, natriuresis, kaliuresis, loss of muscle mass (gastrocnemius and soleus), and increased nitrogenous end products (urine urea and NH_3). These changes follow the patterns previously reported (Musacchia et al. 1980, JAP, 48(3) 479-486 and Deavers et al. 1980, JAP in press).

(2) The rat model described by Musacchia et al. 1980 is being utilized in other laboratories. In order to assess the ready reproducibility of experimentation using this model, a series of studies were undertaken to repeat a portion of our earlier seven day projects. The data for changes in body weight, urine volumes, muscle weight, urine urea and NH_3 excretion in both metabolism cage controls and antiorthostatic hypokinetic animals were comparable with only minimal sporadic differences. Comparisons of data from 1978 (University of Missouri) and 1980 (University of Louisville) experiments are presented.

(3) Examination of our previous data indicates that food and water intakes were depressed in seven day antiorthostatic hypokinetic (AOH) subjects. This raised questions concerning the effect of reduced food and water intakes in AOH individuals. Separate groups of metabolism cage controls were pair fed or pair watered to match the AOH ad libitum consumption. Pair fed and pair watered subjects grew at a rate similar to metabolism cage controls which were ad libitum fed and watered and did not exhibit evidence of diuresis, natriuresis, kaliuresis, muscle atrophy or increased nitrogenous end product excretion. From this evidence it is deduced that the responses to AOH (including: diuresis, natriuresis, kaliuresis, muscle atrophy, depressed growth and elevated urine urea and NH_3 excretion) are not influenced by the ad libitum food and water consumption of suspended subjects but are a result of suspension induced hypokinesia and head-down tilt induced antiorthostasis.